The effects of soil water deficit on physiological, morphological and chemical traits of *Eucalyptus*

by

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Declaration

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This paper was conceived by the **Candidate** and **Authors 2 -5**. **Candidate** conducted the experiment, analysed data and wrote the manuscript. **Author 6** analysed some samples. **Authors 8** and **9** developed the foliar ABA assay and provided guidance on foliar ABA sample preparation, extraction and data interpretation. **Authors 3** and **5** provided guidance with treatments. Modelling of chemical data using Near Infrared Spectroscopy (NIRS) was completed by the **Candidate** in association with **Author 7**. Supervision, guidance and corrections were provided by **Authors 2–5**. **Author 9** also provided feedback on the manuscript.

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Abstract

Drought periods leading to soil water deficit can fundamentally affect plant growth and survival, and alter plant traits such as leaf chemistry. Predictions of increased drought periods in many regions means that widespread plant species may experience soil water deficit that differs in duration and severity over the species natural range. Glasshouse experiments were used to test the effect of water deficit level, water deficit duration and re-watering on juveniles from genetically-distinct *Eucalyptus globulus* and *E. viminalis* provenances by quantifying plant morphological, physiological and chemical traits. *Eucalyptus globulus* provenances vary in genetic-based drought tolerance, and provenances are located across a rainfall gradient. I hypothesised that juveniles of provenances from wet locations would be affected by water deficit to a larger degree than those from dry locations due to intra-specific adaptation to local rainfall patterns.

Both species and all provenances responded similarly to all water deficit treatments and to re-watering regardless of genetic-based constitutive trait variation, local rainfall patterns, or in the case of adult *E. globulus,* field drought susceptibility. The only response to water deficit which varied between provenances was foliar abscisic acid (ABA) levels, and the response pattern was similar for both species at each paired locality, indicating parallel evolution and local adaptation. While many *E. viminalis* constitutive chemical and morphological traits changed over time due to plant development, the duration of moderate (50%) water deficit had no impact on any trait. *Eucalyptus globulus* leaf water potential, foliar ABA levels, plant biomass and C:N were affected by different levels of water availability ranging from 90 – 0% of control evapotranspiration. Plant secondary metabolite (PSM) responses to water deficit varied between experiments, and also between the selected PSM traits. Overall, concentrations of individual terpenes and total oil were not influenced by water deficit. Concentrations of phenols were more plastic in response to water deficit than terpenes, as condensed tannin concentrations increased in some

experiments and total phenolic and formylated phloroglucinol compound (FPC) concentrations decreased in some experiments. A short re-watering period reversed the effects of water deficit on some chemical traits, but also decreased phenol concentrations. Variation in responses to water deficit between experiments is discussed.

Overall, genetic-based quantitative PSM variation was high between species and provenances, with comparably low levels of plasticity due to water deficit. While responses to soil water deficit may largely be similar in juveniles across each species range, the ecological impact of modest changes to *Eucalyptus* PSM concentrations during drought may be equally modest, and the effect on mature trees is unknown.

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Contents

Chapter 1

General Introduction

1.1 Drought and the impact of soil water deficit on plants

Drought is predicted to become more frequent and widespread in many regions (Collins *et al.* 2013). Drought may have severely detrimental effects on plant health (Chaves *et al.* 2003; Lipiec *et al.* 2013; Osakabe *et al.* 2014). For example, soil water deficit can inhibit plant growth (Mokotedi 2010; Pinkard *et al.* 2011), lead to cavitation of conductive cells (Tyree & Sperry 1989; Hacke & Sperry 2001; Cochard *et al.* 2007) and even cause plant death (Runeckles 1982; Brodribb & Cochard 2009). However, plants employ strategies to minimise within-plant water loss, thereby limiting the detrimental effects of soil water deficit (McDowell *et al.* 2008). Plants can regulate levels of within-plant water using stomatal control, as closing stomata limits foliar water loss and decreases the potential for tissue desiccation (Chaves *et al.* 2003). Plants reduce stomatal aperture using the phytohormone abscisic acid (ABA), as high foliar ABA levels reduce guard cell turgor, closing stomata, which inhibits transpiration (Tardieu & Simonneau 1998; Bauer *et al.* 2013; McAdam & Brodribb 2014) in response to high vapour pressure deficit (McAdam & Brodribb 2015b). However, once stomata are closed, within-plant water potentials (Ψ; a measure of vascular tension) become increasingly negative as the Ψ of soil becomes more negative (Brodribb & McAdam 2011).

While the closure of stomata reduces plant water loss, it also limits carbon dioxide (CO_2) intake and, therefore, the ability of plants to photosynthesise (McDowell *et al.* 2008; Brodribb & Cochard 2009). Photosynthesis produces carbohydrates which are vital for plant growth and cell differentiation, yet reduced

carbon assimilation during soil water deficit can lead to a negative within-plant carbon balance and a reduced pool of available carbohydrates (McDowell *et al.* 2008; Mitchell *et al.* 2014). The biosynthesis of plant chemical compounds requires an adequate pool of carbohydrates, and so chemical biosynthesis may also be inhibited by water deficit and a depleted within-plant carbon store. Theory suggests that moderate water deficit which inhibits growth more than photosynthesis will increase phytochemical concentrations (Herms & Mattson 1992). This predicted response is based on the high levels of water required for cell elongation and division compared to photosynthesis, and the pool of carbohydrates made available for other roles such as phytochemical biosynthesis when plant growth is limited (Herms & Mattson 1992). It is this link between environment (variation in water availability) and the allocation of limited resources to growth and phytochemistry which is tested in the experimental chapters of this thesis.

1.2 Plant secondary metabolites

Traditionally, chemical traits required for plant growth and cellular differentiation are known as primary metabolites, while compounds not fundamentally required for plant growth and development are termed secondary metabolites (Fraenkel 1959). The concentrations of plant secondary metabolites (PSMs) are under genetic control (Berenbaum *et al.* 1986; Simms & Rausher 1987; Barton *et al.* 1991; Doran & Matheson 1994), vary with ontogeny (Barton & Koricheva 2010; Borzak *et al.* 2015), and with environmental conditions (McArthur *et al.* 2003; Laitinen *et al.* 2005; Close *et al.* 2007; Glynn *et al.* 2007; Bidart-Bouzat & Imeh-Nathaniel 2008; Lindroth 2010; Julkunen-Tiitto *et al.* 2014). For example, PSM concentrations can change seasonally (Steinbauer *et al.* 2015) or due to nutrient availability (Lawler *et al.* 1997; Hyvarinen *et al.* 2003; O'Reilly-Wapstra *et al.* 2005), atmospheric CO_2 concentration (Kinney *et al.* 1997; Lawler *et al.* 1997; Lindroth *et al.* 2002; Couture *et al.* 2014) and soil water availability (Leicach *et al.* 2010; Pizarro & Bisigato 2010; Yadav *et al.* 2014). As such, the concentration of

PSMs in plant tissues varies due to the interaction between plant genotype and different environmental factors (Laitinen *et al.* 2005).

PSMs are grouped into chemical classes defined by common biosynthetic pathways, the common structure of compounds (Wink 2010) and also by compound properties (e.g. tannins; Ferreira *et al.* 2008). There are three major PSM groups; the terpenoids and the phenolics which are carbon-based compounds and contain no nitrogen, and the third group are the nitrogen-containing compounds. The chemical structure of many of these compounds and the biosynthetic pathways involved in producing the diverse array of PSMs within these three groups have been detailed elsewhere (Cowan 1999; Herrmann & Weaver 1999; Eschler *et al.* 2000; Heim *et al.* 2002; Moore *et al.* 2004; Keller *et al.* 2005; Prescher & Bertozzi 2005; Zhao *et al.* 2005; Keszei *et al.* 2008; Crozier *et al.* 2009; Keszei *et al.* 2010; Petersen *et al.* 2010; Vogt 2010; Salminen & Karonen 2011) and are outside the scope of this thesis. This thesis focuses on the effect of water deficit on concentrations of selected individual compounds and bulk compound groups of phenolics and the terpenes. I focus on these non-nitrogen containing PSMs, as with the exception of the nitrogen-containing cyanogenic glycosides (Gleadow & Woodrow 2002; Woodrow *et al.* 2002; Vandegeer *et al.* 2013; Kooyers *et al.* 2014), previous studies testing the effects of water deficit on PSM concentrations have largely quantified phenolics and terpenes (see below). I aimed to build upon previous work by quantifying some of these same PSMs, but expanding the diversity of water deficit treatments and the range of plant genetic material used.

Firstly I will provide a brief summary of the terpenoids and the phenolics, the two non-nitrogen containing PSM groups, and then provide an overview of the known ecological roles of these PSM groups. Secondly, I will introduce the genus *Eucalyptus* along with inter- and intra-specific variation of *Eucalyptus* traits. Thirdly, I will summarise the literature regarding water deficit effects on terpenes and

phenolics in order to find trends in the responses. Specifically, I begin with an overview of terpene and phenolic responses to water deficit among different plant forms, then I focus on responses in woody eudicots (including eucalypts), and then discuss the impact of water deficit level on the associated PSM response. Finally, I will state the aims of this thesis and provide an overview of experimental chapters.

1.2.1 Terpenoids

Terpenes are the largest group of PSMs, with over 36 000 individual terpenoid compounds having been reported (Buckingham 2007). All terpenes are produced from the initial biosynthesis of C_5 units, either isopentenyl diphosphate (IPP; C_5) or dimethylallyl diphosphate (DMAPP; C_5), along the mevalonic acid or methylerythritol phosphate pathways (Dudareva *et al.* 2004; Ashour *et al.* 2010). IPP and DMAPP condense to form geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate. Next, various prenyl diphosphates, DMAPP (C_5), GPP (C_{10}), FPP (C_{15}) and geranylgeranyl diphosphate (C_{20}) are converted to hemiterpenes (isoprene and 2-methyl-3-buten-2-ol), monoterpenes (C_{10}), sesquiterpenes (C_{15}), and diterpenes (C_{20}), respectively (McGarvey & Croteau 1995; Bohlmann *et al.* 1998; Dudareva *et al.* 2004). These transformations are catalysed by the terpenoid synthases (cyclases), and are followed by a variety of redox modifications of the parent skeletal types to produce the vast number of individual terpenes (Buckingham 2007).

The location of terpene synthesis differs within cells depending on the terpene class. While some components of terpene biosynthesis occur in the cytoplasm, there is evidence that monoterpene and diterpene synthesis occurs in the chloroplast, while sesquiterpenes are synthesised in the endoplasmic reticulum (Roberts 1981; Wink & Hartmann 1982; Kutchan 2005). Many terpenoids are stored in specialised plant structures, including resin ducts in wood and leaves of conifers,

glandular trichomes, and oil glands in the leaves of certain taxa (Fahn 1979). This project uses *Eucalyptus* as a model species, and oil glands in leaves of Myrtaceae (including *Eucalyptus*) contain oil consisting primarily of mono- and sesquiterpenes (e.g. Boland *et al.* 1991; Li *et al.* 1996; McKiernan *et al.* 2012), yet low concentrations of other PSMs (e.g. phenols) can also be quantified in *Eucalyptus* oil samples (e.g. McKiernan *et al.* 2012). The most abundant components of *Eucalyptus* oil vary among species (Boland *et al.* 1991; Brophy & Southwell 2002), but the monoterpene 1,8-cineole is often a large proportion of the total oil (Li *et al.* 1995; Li *et al.* 1996; Pass *et al.* 1998; King *et al.* 2004).

1.2.2 Phenolics

The second major group of PSMs investigated in this thesis are the phenolics. The phenolics are diverse, and include the flavonoids, tannins, phenylpropanoids, lignin, lignans, coumarins and polyketides (Wink 2010). In the general phenyl-propanoid pathway of phenolic biosynthesis, L-phenylalanine (and to a lesser extent L-tyrosine) is transformed to coenzyme A-activated 4-coumaric acid (4-coumaroyl-CoA) by cinnamic acid 4-hydroxylase, phenylalanine ammonia-lyase and 4-coumarate CoA-ligase (Petersen *et al.* 2010). 4-Coumaroyl-CoA then gives rise to the wide range of phenolic classes via further biosynthesis (Petersen *et al.* 2010), which is complex, beyond the scope of this thesis, and detailed by other workers (e.g. Cowan 1999; Heim *et al.* 2002; Keller *et al.* 2005; Zhao *et al.* 2005; Crozier *et al.* 2009; Petersen *et al.* 2010; Vogt 2010). In contrast to sites of terpene (lipophilic) storage, phenolics are generally hydrophilic and are often stored in vacuoles (Kutchan 2005). In the experimental chapters of this thesis, I quantify selected individual phenolic compounds and two broad phenolic groups (total phenolics and condensed tannins).

The two bulk phenolic PSM traits that are most commonly assayed by ecologists are the total phenolics and the condensed tannins (e.g. Iason *et al.* 1993;

Lawler *et al.* 1998; Gleadow & Woodrow 2002; Glynn *et al.* 2004; Milam *et al.* 2004; Ishida *et al.* 2008; Hofland-Zijlstra & Berendse 2009; Pizarro & Bisigato 2010; Zhang *et al.* 2012). Quantification of 'total phenolics' in a biological sample provides a concentration of total unknown phenols, which may include any number of individual compounds from across the phenol classes (e.g. tannins, flavonoids, coumarins). The second bulk phenol assay involves the condensed tannins. The tannins are structurally diverse, and are characterised by the ability to bind and precipitate proteins (Petersen *et al.* 2010). Tannins can be subdivided by structure into the hydrolysable tannins (esters of gallic acid) and the condensed tannins of flavonoid origin (Petersen *et al.* 2010). Here, I focus on the condensed tannins (proanthocyanidins). Assaying condensed tannins again provides a bulk quantification of an unknown number of compounds, each of unknown concentration.

Aside from the bulk phenol assays, I include quantification of individual compounds. Phloroglucinols are phenol derivatives, and formylated phloroglucinol compounds (FPCs) are mono to tetra-formylated phloroglucinol base derivatives with an attached terpene moiety (Eschler *et al.* 2000) in most cases. FPCs appear limited to foliage of *Eucalyptus* from the sub-genus *Symphyomyrtus* (Eschler *et al.* 2000). Here, I quantify two macrocarpals (A and G) and a sideroxylonal (A), as these FPCs have been identified from a number of *Eucalyptus* species and have previously been shown to have ecological influences (see below). Biosynthesis of macrocarpals A and G involves the sesquiterpene bicyclogermacrene (Pass *et al.* 1998), whereas sideroxylonal A is formed of two isopentyl diformyl phloroglucinols without a terpene moiety (Satoh *et al.* 1992).

1.2.3 Ecological roles of plant secondary metabolites

In this thesis I do not directly test the effect of PSMs on biota, biotic interactions or ecosystem processes. However, many roles of PSMs are known (and

many are unknown) and changes to PSM concentrations due to water deficit (as tested in this thesis) may influence or alter these roles. For example, PSMs can affect the composition of invertebrate communities associated with plant genotypes (Barbour *et al.* 2009b; Barbour *et al.* 2015). Phenolics relieve oxidative stress within plants cells during water deficit (Sgherri *et al.* 2004) and cause oxidative stress in herbivores (Salminen & Karonen 2011). Phenols can influence soil microbial activity and microbial community composition (Ushio *et al.* 2013), and tannins bind with proteins and inhibit digestion of plant material by herbivores (Foley & Hume 1987). FPCs are known to deter mammal and insect browsing (Lawler *et al.* 1998; Lawler *et al.* 1999a; e.g. Moore *et al.* 2004; O'Reilly-Wapstra *et al.* 2004; Wiggins *et al.* 2006a; Andrew *et al.* 2007a), affect marsupial tree selection (Youngentob *et al.* 2011) and also have other influences such as marine anti-fouling properties (Singh *et al.* 1996).

Many terpenes are volatile (Kesselmeier & Staudt 1999), and the primary functions of airborne volatiles are to defend plants against herbivores and pathogens or to provide a reproductive advantage by attracting pollinators and seed dispersers (Dudareva *et al.* 2006). The concentration of the total oil in leaves is linked with fire regimes (Steinbauer 2010) and high oil content of leaf litter increases litter flammability (Alessio *et al.* 2008a; Alessio *et al.* 2008b; Ormeño *et al.* 2009). High oil content of leaves also decreases litter decomposition rates (Horner *et al.* 1988; Tharayil *et al.* 2011; Chomel *et al.* 2014). Specific terpenes also have known roles. For example, high foliar concentrations of 1,8-cineole decrease herbivory by mammals (Wiggins *et al.* 2003), yet 1,8-cineole has also been identified as an important olfactory cue for mammals during foraging (Bedoya-Pérez *et al.* 2014). α-Pinene is an invertebrate attractant (Nordlander 1990; Branco *et al.* 2010), which also can inhibit beetle pheromone effectiveness (Erbilgin *et al.* 2003) and can reduce nitrification in soil (Paavolainen *et al.* 1998). For reviews discussing further ecological roles of terpenes see Langenheim (1994) or Gershenzon and Dudareva (2007).

1.3 *Eucalyptus*

1.3.1 The genus Eucalyptus

As mentioned above, this thesis focuses on the genus *Eucalyptus*. *Eucalyptus* is a dominant tree genus consisting of over seven hundred species divided between seven polytypic and six monotypic subgenera (Brooker 2000; Steane *et al.* 2011). Most *Eucalyptus* species are endemic to Australia, where they are often dominant in the landscape (Brooker 2002). Eucalypts grow naturally across many environmental gradients including rainfall, temperature and altitudinal gradients, and species fill a range of environmental niches (Pryor 1976; Williams & Potts 1996; Williams & Woinarski 1997; Reid & Potts 2005; Givnish *et al.* 2014). For example, *E. gunnii* and *E. pauciflora* are frost tolerant subalpine species (Davidson & Reid 1985), whereas the mallee *Eucalyptus* (which grow from lignotubers with multiple stems and narrow canopy zone) are found in semi-arid regions and are tolerant to prolonged drought (Noble 2001). The ability to tolerate water deficit varies between *Eucalyptus* species, and may be linked to species growth-rate and water uptake (Lewis *et al.* 2013). The natural range of individual *Eucalyptus* species can overlap at ecotones (Battaglia & Williams 1996). Apart from these ecotonal regions, mixed stands containing multiple co-dominant *Eucalyptus* species from different subgenera (e.g. *E. obliqua* or *E. tenuiramis* with *E. viminalis*) are found in some regions of Australia (Duff *et al.* 1983; Noble 1989). Furthermore, interbreeding among species from the same subgenus with overlapping ranges can result in continuous clinal variation between recognised species (Potts & Wiltshitre 1997).

1.3.2 Variation in Eucalyptus *traits*

Given the large natural ranges of some eucalypt species, plant traits can differ across a species' range. For example, morphological traits such as oil gland density, bark thickness and leaf length can vary between genetically-differentiated provenances (Ladiges *et al.* 1981; Dutkowski & Potts 1999). In addition, genetically-determined intra-specific drought tolerance (Ladiges 1974; Dutkowski & Potts 2012)

and quantitative phytochemical variation exists between provenances within *Eucalyptus* species (Andrew *et al.* 2010; O'Reilly-Wapstra *et al.* 2010; Wallis *et al.* 2011). Intra-specific variation in constitutive PSM concentrations may have resulted from diversifying selection driven by local selection pressures (Storz 2005; Strand *et al.* 2012; Abdellaoui *et al.* 2013), including variation in environmental factors. Given the existence of constitutive trait variation among provenances of a species, provenances may also have evolved to have different responses to environmental conditions. Such provenance-specific plasticity has been reported for growth, leaf morphology or physiology in response to varying levels of water availability in field (McLean *et al.* 2014) and glasshouse (Gibson *et al.* 1995; Li & Wang 2003) trials of *Eucalyptus*. Consequently, a key aim of this PhD research was to test PSM plasticity during soil water deficit among provenances within *Eucalyptus* species.

Eucalyptus leaves generally contain high levels of phenols and terpenes, yet FPCs are absent from the leaves of the *Eucalyptus* subgenus, *Eucalyptus* (Eschler *et al.* 2000). The individual PSM compounds in eucalypt leaves vary qualitatively and quantitatively between *Eucalyptus* species (Li *et al.* 1996; Nicolle *et al.* 1998; Steinbauer 2010; Steinbauer *et al.* 2015). For example, *E. grandis* leaves contain high concentrations of α-pinene (Henery *et al.* 2008), whereas *E. camaldulensis*, *E. globulus* and *E. viminalis* leaves contains high concentrations of 1,8-cineole (Doran & Bell 1994; Leicach *et al.* 2010). In fact, foliar constitutive concentrations of 1,8-cineole and α-pinene are negatively correlated within many eucalypt species (Steinbauer 2010; Borzak *et al.* 2015), yet not all investigations describe this correlation (O'Reilly-Wapstra *et al.* 2011). PSM concentrations also vary within populations among neighbouring trees (Wallis *et al.* 2002; Neilson *et al.* 2006; Kennington 2009; O'Reilly-Wapstra *et al.* 2013; Marsh *et al.* 2014; Stalenberg *et al.* 2014; Steinbauer *et al.* 2015), and even among leaves of different branches of a single tree (Padovan *et al.* 2012) creating a chemical mosaic in the landscape.

Compared to the number of studies that have quantified PSM concentrations in *Eucalyptus* species (e.g. Nicolle *et al.* 1998; Steinbauer 2010; Heskes *et al.* 2012), intra-specific variation in PSMs has only been investigated in a relatively small number of commercially important or widespread *Eucalyptus* species (Lawler *et al.* 1998; Wallis *et al.* 2002; O'Reilly-Wapstra *et al.* 2004; Andrew *et al.* 2007b; Andrew *et al.* 2010; O'Reilly-Wapstra *et al.* 2010; Wallis *et al.* 2011; Heskes *et al.* 2012; McKiernan *et al.* 2012; Andrew *et al.* 2013; O'Reilly-Wapstra *et al.* 2013; Borzak *et al.* 2015). Given the important and varied roles of different PSMs, changes to PSM concentrations caused by drought may have tangible consequences in an ecosystem, and the consequences may differ geographically if plastic responses vary among provenances within a species. Even if eucalypt provenances from across a species range were to respond similarly to soil water deficit, the actual response is difficult to predict based on current published findings (see below).

1.4 Impacts of water deficit on PSM concentrations

1.4.1 Published accounts of PSM variation

The impact of water deficit on foliar PSM concentrations varies widely among studies, plant species, PSM classes and levels of water deficit (Supplemental Tables S1 and S2). As introduced above, soil water deficit limits the availability of water for plant uptake, but also reduces nutrient uptake, within-plant transport of resources, foliar CO_2 intake and photosynthesis (McDowell *et al.* 2008). Therefore, plant growth and the synthesis of PSMs should also be negatively affected by soil water deficit. However, theories of resource allocation predict that moderate water stress, which inhibits growth more than photosynthesis, would result in increased allocation to chemical defence (Herms & Mattson 1992). Following this hypothesis, further decreases in water availability would inhibit both growth and photosynthesis, thereby limiting PSM biosynthesis (Herms & Mattson 1992). Contrastingly, a review of medicinal plant responses to water stress demonstrated that levels of antioxidant compounds (e.g. phenols) increased even after stomatal closure (Selmar &

Kleinwächter 2013), possibly through utilisation of pre-existing within-plant carbon stores.

PSM responses to water deficit not only differ because of the size of water deficit effects (i.e. small or large concentration changes), but differ because of opposing effects (i.e. increased, decreased or no effect on concentrations) on the concentrations of individual PSMs (Supplemental Tables S1 and S2). While no previous study has investigated the effect of water deficit on FPC concentrations in any species, a number of published articles have reported water deficit impacts on total oil yield and individual terpene concentrations, or on phenol concentrations across a range of taxa (Fig. 1a-d; Supplemental Tables S1 and S2). Most studies (n=81) have focussed on PSM responses in woody eudicots (Fig. 1b), with very little work (n=4) reporting PSM concentrations within conifer foliage (Fig. 1d). In conifers, it is more common for the emission of volatile terpenes from stomata to be quantified than the quantification of terpenes levels within needles (e.g. Peñuelas & Llusià 1999; Niinemets *et al.* 2002; Blanch *et al.* 2007; Ormeño *et al.* 2007; Llusià *et al.* 2008; Blanch *et al.* 2009; Lusebrink *et al.* 2011). As the levels of volatile terpene emissions from stomata are not directly comparable with terpene concentrations within needles, these studies have not been included here. Over all the reports reviewed, soil water deficit had no statistically significant impact on PSM concentrations 47-56% of the time (Fig. 1). When water deficit did impact PSM concentrations, water deficit increased PSM concentrations (32-50% of reports) more often than it decreased concentrations (0-21% of reports; Fig. 1).

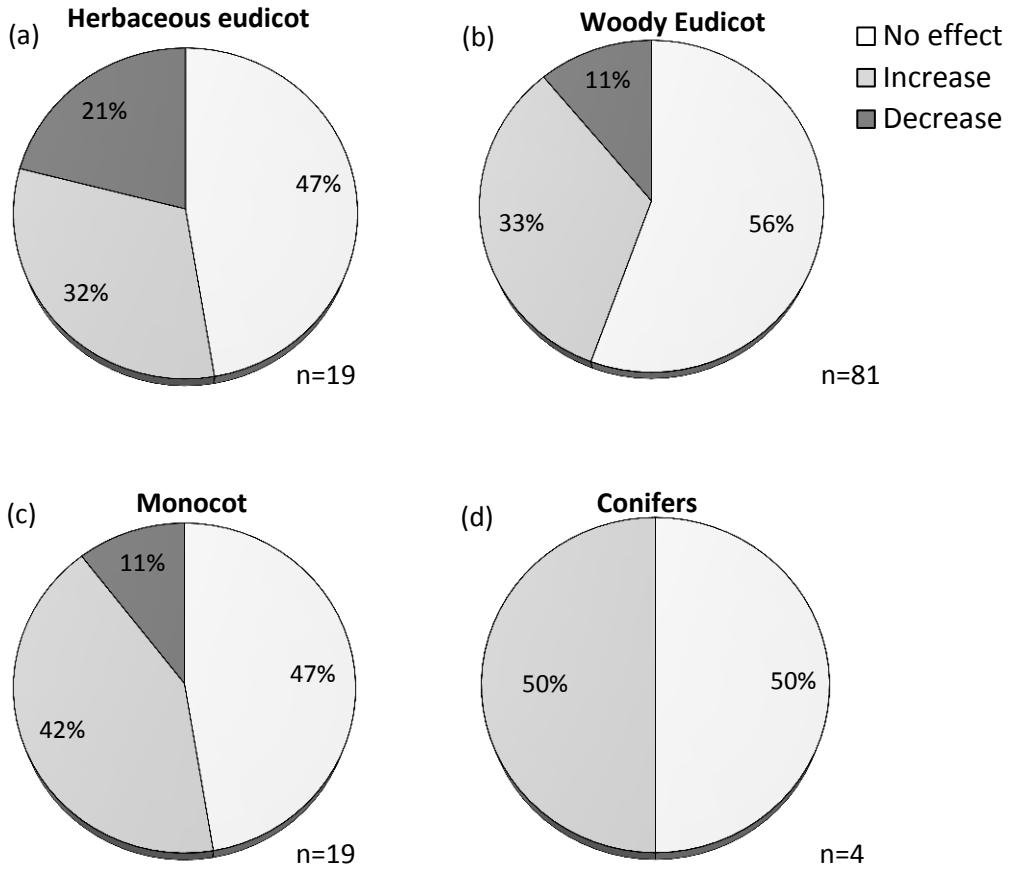

Figure 1. Effects of soil water deficit on the concentrations of plant secondary metabolites (combined terpenes and phenols) in four plant types. Percentages in each segment indicate percentage of total reports of each response. The total number of reports (*n*) is given for each graph, taken from Supplemental Tables S1 and S2. *No effect* indicates that no statistically significant effect of water deficit was detected in that journal article. Concentration of terpenes and phenols in each report were usually expressed as mg g^{-1} DM using standards.

1.4.2 Woody eudicots

Across the large number of studies (n=81) on woody eudicots, concentrations of terpenes and the total oil yield were affected (increased or decreased concentrations) more commonly by water deficit (83%; Fig. 2a) than concentrations of phenols (32%; Fig. 2b). Of these changes, terpene concentrations in woody eudicots primarily increased (67% of reports) while very few studies found that terpene concentrations remained stable during water deficit (17%; Fig. 2a). In contrast, phenol concentrations were less plastic in response to water deficit, as most previous studies reported no influence of water deficit on phenol concentrations (68%; Fig. 2b). When phenols did respond to water deficit (32% of reports in total), concentrations were more likely to increase (22% of reports) than decrease (10%; Fig. 2b).

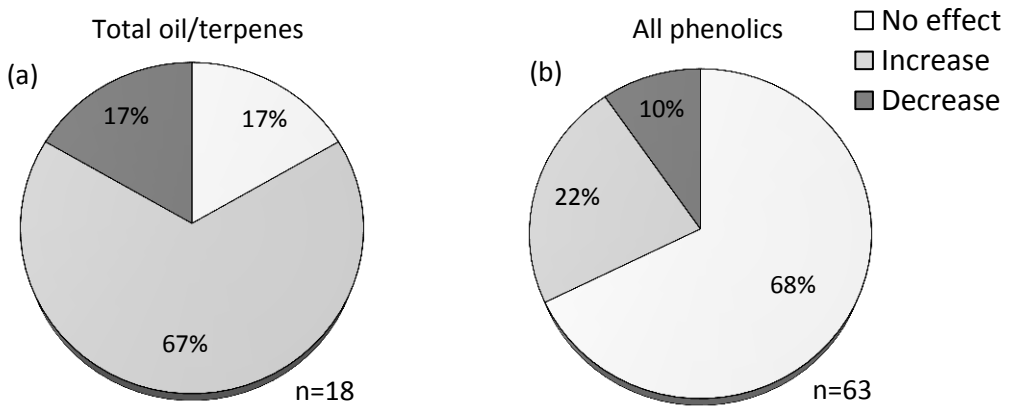

Figure 2. Effects of soil water deficit on concentrations of plant secondary metabolites in woody eudicots grouped into (a) total oil yield and individual terpenes and (b) phenols including tannins. No previous study has investigated the effect of water deficit specifically on formylated phloroglucinol compounds. Percentages in each segment indicate percentage of total reports of each response. The total number of reports (*n*) is given for each graph, taken from Supplemental Tables S1 and S2. *No effect* indicates that no statistically significant effect of water deficit was detected in that journal article. Concentration of terpenes and phenols in each report usually expressed as $mg.g^{-1}$.DM using standards.

1.4.3 Link between the level of water deficit and the change in PSM concentrations

In order to gauge how different levels of water deficit may impact PSM concentrations in eudicots, data from Supplemental Tables S1 and S2 which describe a statistically significant effect of water deficit on phenolic and terpene concentrations (i.e. excluding 'no effect' data) were selected. The level of water deficit (% of control water) was compared against the effect size reported (% increase or decrease in concentration; Fig. 3) in order to visualise the response trend without statistical analysis. The effect size of water deficit on PSM concentrations in woody eudicots appears to positively correlate with the level of water deficit applied in experimental treatments (Fig. 3). Specifically, PSM concentrations in eudicots subjected to mild water deficit (c. 60 -75% of control water) quantitatively changed (either increased or decreased) by around 50%. Contrastingly, PSM concentrations in eudicots subjected to severe water deficit (c. 25% of control water) changed by up to 260% (Fig. 3).

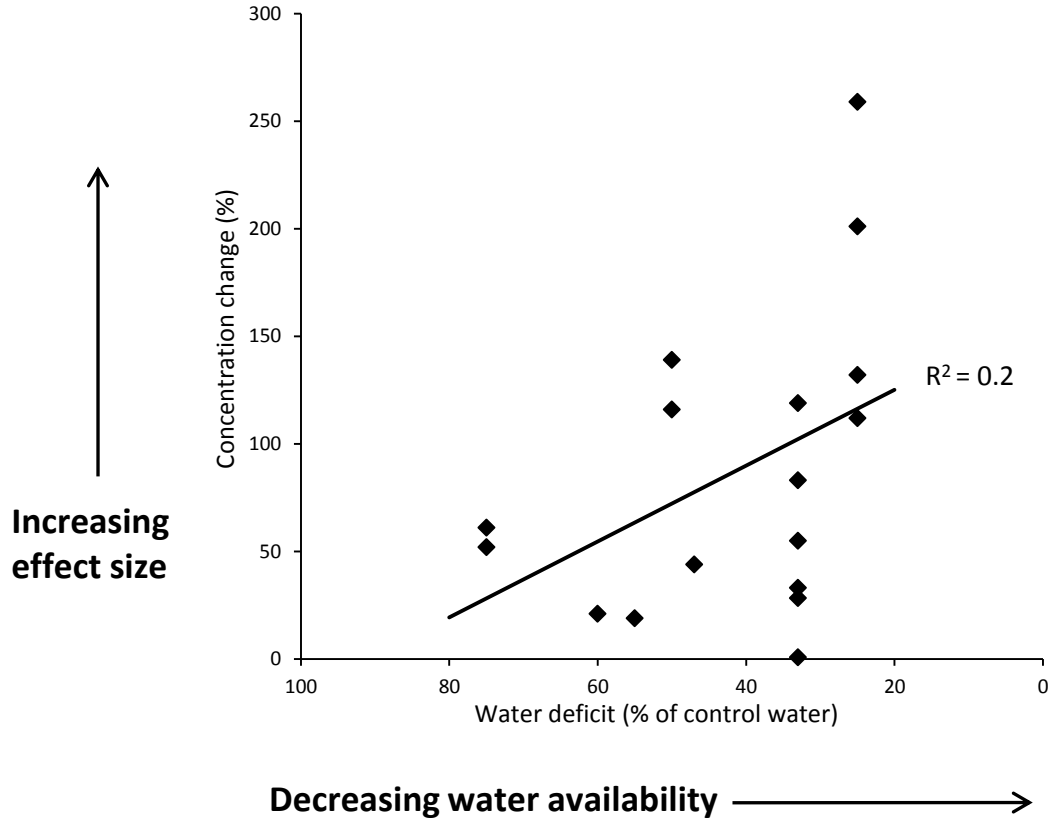

Figure 3. Relationship between decreasing water availability and the magnitude of changes to plant secondary metabolite (PSM) concentrations in woody eudicots. Data taken from published literature (Supplemental Tables S1 and S2).

A number of conclusions and trends regarding the effect of water deficit on PSM concentrations can be drawn from the literature. First, PSM responses to water deficit vary among plant forms (Fig. 1). Second, within the woody eudicots, terpene concentrations appear more plastic than phenol concentrations in response to water deficit (Fig. 2). Finally, the level of water deficit applied to woody eudicots appears to influence the effect size on PSM concentrations (Fig. 3).

Relatively few studies have quantified the influence of soil water deficit on the concentrations of foliar PSMs (Supplemental Tables S1 and S2). This could be considered a dearth in the literature considering the number of studies which have characterised and quantified PSMs (Cowan 1999; Herrmann & Weaver 1999; Heim *et al.* 2002; Keller *et al.* 2005; Prescher & Bertozzi 2005; Zhao *et al.* 2005; Crozier *et al.* 2009; Vogt 2010 and references within), the large array of ecological interactions that PSMs influence (Horner *et al.* 1988; Bryant *et al.* 1991; Rosenthal & Berenbaum 1992; Luo *et al.* 2007; Alessio *et al.* 2008b; Bidart-Bouzat & Imeh-Nathaniel 2008; Barbour *et al.* 2009b; Ormeño *et al.* 2009; Bardon *et al.* 2014; Chomel *et al.* 2014), and the predicted increase in drought frequency and severity in many regions (Collins *et al.* 2013). Here, using juvenile woody eudicots of the genus *Eucalyptus*, I investigate the interaction between water availability and PSM concentrations to shed light on the existing response variability in the literature.

Apart from their ecological and economic significance, eucalypts are an ideal study system because (1) of the expanding knowledge base on the effects of water deficit on the physiology of individual *Eucalyptus* plants (e.g. Osório *et al.* 1998; Pita & Pardos 2001; Costa e Silva *et al.* 2004; Shvaleva *et al.* 2006; Battie-Laclau *et al.* 2014; Martorell *et al.* 2014; Mitchell *et al.* 2014; White *et al.* 2014) and, (2) much is already known about the diversity and concentrations of PSMs in *Eucalyptus* (e.g. Wallis *et al.* 2002; O'Reilly-Wapstra *et al.* 2004; Wiggins *et al.* 2006c; Andrew *et al.* 2007a; Keszei *et al.* 2008; O'Reilly-Wapstra *et al.* 2010; O'Reilly-Wapstra *et al.* 2011), their population genetics (e.g. Li 2000; Li *et al.* 2000; Jones *et al.* 2002;

Gleadow *et al.* 2008; Bloomfield *et al.* 2011; Steane *et al.* 2011; Wallis *et al.* 2011) and the broad-scale influence of drought on *Eucalyptus* stands and populations (e.g. Ladiges 1974; White *et al.* 1996; Susiluoto & Berninger 2007; Tausz *et al.* 2008; Dutkowski & Potts 2012; Warren *et al.* 2012; Zeppel *et al.* 2012; Héroult *et al.* 2013). In this thesis, I use common environment glasshouse trials to test for genetic-based differences in responses to soil water deficit at the species-level between *Eucalyptus globulus* and *E. viminalis,* but also among within-species provenances. Furthermore, as these two *Eucalyptus* species often co-occur, paired sampling was undertaken at multiple locations to enable response trend confirmation across species, and to test for parallel evolution of responses across species. No previous study has tested the responses of a range of PSMs in multiple provenances of two species to different levels and durations of water deficit. I hypothesised that provenances from wet localities would experience greater water stress, and that traits would be quantitatively altered to a greater degree by a particular level of water deficit compared to provenances from drier localities. Based on the literature, I also hypothesised that the levels of terpenes would be more plastic in response to soil water deficit than the levels of phenols.

1.5 Aims

This thesis had four major aims, which were to:

1. investigate the effect of moderate and severe water deficit on concentrations of select non-nitrogen containing PSMs (terpenoids and phenolics) among leaf age classes within individual *E. globulus* and *E. viminalis* plants;

2. test intra-specific responses to moderate water deficit and to re-watering (recovery) using paired *E. globulus* and *E. viminalis* provenances from four environmentally- and geographically-distinct localities;

3. examine the influence of water deficit duration and re-watering on specific morphological, physiological and chemical traits of juvenile *E. viminalis*; and

4. explore how the intensity of water deficit affected juvenile *E. globulus* traits by subjecting plants to one of eleven levels of water availability.

1.6 Overview of chapters

Chapter 2 reports an initial glasshouse experiment to investigate the impact of two levels of soil water deficit on plant morphological and chemical traits of juvenile *Eucalyptus globulus* and *E. viminalis* leaves divided into three within-plant foliage age classes. This experiment was designed as an initial test to gauge the responses of these eucalypts to soil water deficit, to identify the aspects of water deficit that may induce different responses in plant traits, and to test the technique for maintaining soil water deficit.

Chapter 3 describes a large glasshouse-based experiment which investigated genetic-based variation in juvenile *E. globulus* and *E. viminalis* responses to water deficit and recovery between species and native provenances within-species. Provenances within each species were paired such that both species were sampled from the same general locality in the wild, with the four sampled localities differing in their rainfall patterns. These provenances have quantitatively diverse constitutive traits, and a previous study showed that mature *E. globulus* from these locations have different levels of drought tolerance.

Chapter 4 focuses on the effect of water deficit duration on juvenile *E. viminalis* from the four provenances, and the interaction between the duration of water limitation and plant recovery ability after water limitation. I tested whether short term soil water deficit (e.g. 12 days) would impact plant traits differently to a longer period of water deficit (e.g. 66 days).

Chapter 5 is the final experimental chapter, and questions the level of water deficit required to alter physiological, morphological and chemical traits of juvenile *E. globulus*. *Eucalyptus globulus* were grown under one of eleven different levels of water availability ranging from fully watered (field capacity) down to completely unwatered.

Chapter 2

Effect of limited water availability on foliar plant secondary metabolites of two *Eucalyptus* species

This chapter is published as:

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2.1 Abstract

Plant secondary metabolites (PSMs) have many ecological roles such as influencing decomposition, flammability and herbivory. PSM concentrations are genetically determined, but are also affected by environmental factors. Drought periods are predicted to become more frequent in many regions, and may have widespread impacts on PSM-mediated ecological interactions. We used two dominant tree species to investigate the impact of multiple levels of water availability on leaf PSM concentrations. Juvenile *Eucalyptus globulus* and *E. viminalis* plants were subjected to one of three glasshouse watering regimes (high [control], moderate or low water availability) and the effect on leaf PSMs was investigated between species, treatments, and within-plant foliage age classes. Moderate and low water availability decreased total phenolic concentrations of both species, and decreased the C:N of *E. globulus* leaves. Low water availability reduced the concentrations of two terpene compounds, but only in specific foliage age classes. Overall, the majority of terpenes were unaffected by decreased water availability, as were two formylated phloroglucinol compounds and condensed tannins. We conclude that water limitation had little impact on overall leaf PSM concentrations, and that juveniles of these two eucalypt species generally maintained PSM concentrations while plant growth declined, eluding to both direct impacts of water limitation and within-plant resource prioritisation.

2.2 Introduction

Plant secondary metabolites (PSMs) play diverse ecological roles including mediating plant/herbivore (O'Reilly-Wapstra *et al.* 2004; Wiggins *et al.* 2006a) and tri-trophic level interactions (Cory & Hoover 2006), promoting plant flammability (Ormeño *et al.* 2009) and influencing rates of leaf litter decomposition (Lindroth 2010). Many of these and other examples in the literature show that the effects of PSMs can extend past the traditional phenotype of the individual and affect dependent communities and ecosystem processes (Whitham *et al.* 2003; Lindroth 2010; Whitham *et al.* 2012). Changes to PSM concentrations can occur due to variation in a range of environmental conditions including nutrients (Gleadow & Woodrow 2002), carbon dioxide (CO_2), ozone (O_3) (Peltonen *et al.* 2010), light (Mooney *et al.* 2009) and temperature (Wahid *et al.* 2007; Jamieson *et al.* 2015). For example, increased temperature can lead to up-regulation of PSM synthesis (Wahid *et al.* 2007), and nitrogen availability affects concentrations of phenolics (Gleadow & Woodrow 2002; O'Reilly-Wapstra *et al.* 2005). Furthermore, the quantity of a resource that is available to a plant (e.g. high, intermediate and low availability) can differentially affect the concentration of individual PSMs (Gutbrodt *et al.* 2012a). As such, experiments utilising multiple levels of resource availability will be more informative than experiments investigating the effect of a single resource level (i.e. control and one experimental treatment) on PSM concentrations.

Drought is receiving increased attention in the literature due to the impact of recent drought periods (Choat *et al.* 2012), and the predicted decrease in rainfall across many regions (Meehl *et al.* 2007). Water limitation is problematic for plants as it inhibits plant access to resources used in photosynthesis, due to stomatal closure and reduced within-plant water transport (Bréda *et al.* 2006). As such, when water is limited plants elicit morphological, physiological and biochemical changes in order to tolerate water stress, or because water deficit has impaired normal plant function (Blackman *et al.* 2009; Mitchell *et al.* 2013). Changes to plant biochemical profiles

may have significant effects outside the plant, especially if the plant species is dominant in the community (Whitham *et al.* 2012). Yet, despite the ecological consequences of changes to plant biochemical traits, surprisingly little is known about the effects of water stress on PSM expression in many dominant plant species.

Eucalyptus is a species-rich tree genus in Australia that commonly dominates the continent's woodlands and forests (Brooker 2002). Eucalypt foliage contains a diversity of PSMs that vary qualitatively and quantitatively between species (Li *et al.* 1996; McKiernan *et al.* 2012; Moles *et al.* 2013). PSM concentrations are also known to vary between leaves on individual eucalypt trees, and this is often associated with leaf age (Silvestre *et al.* 1997) and ontogenetic stage (O'Reilly-Wapstra *et al.* 2007). Plant secondary metabolites in eucalypts influence many ecological interactions. For example, formylated phloroglucinol compounds (FPCs) are important anti-feedants against marsupial herbivores (Wiggins *et al.* 2006a; O'Reilly-Wapstra *et al.* 2010; Youngentob *et al.* 2011), essential oil concentration affects plant flammability (Holmes 2009) and phenolics influence litter decomposition (Horner *et al.* 1988). However, few studies have investigated the effects of reduced water availability on PSM concentrations in *Eucalyptus* (Doran & Bell 1994; Stone & Bacon 1994; Gleadow & Woodrow 2002; King *et al.* 2004; Leicach *et al.* 2010). Most of this work has targeted a single PSM class (e.g. terpenes) and, to our knowledge, no previous study has linked multiple PSM classes in eucalypts to multiple levels of water availability.

Here, we use juvenile *E. globulus* Labill. and *E. viminalis* Labill. to investigate the effect of three levels of glasshouse-based water availability on a subset of non-nitrogen containing foliar PSMs. *Eucalyptus globulus* and *E. viminalis* are classified in sub-genus *Symphyomyrtus*, section *Maidenaria* (Brooker 2000) and are, therefore, phylogenetically closely related within the diverse genus *Eucalyptus*. *Eucalyptus globulus* is of interest as it is dominant in forest stands in south-eastern

Australia and is the most widely planted hardwood species in temperate regions of the world (Potts *et al.* 2004). Understanding *E. globulus* responses to water limitation is, therefore, of global importance. *Eucalyptus viminalis* is also widespread throughout south-eastern Australia (Williams & Potts 1996) and both species are important food sources for the regionally vulnerable koala (*Phascolarctos cinereus*) (Moore & Foley 2005; Moore *et al.* 2005). *Eucalyptus viminalis* was selected due to its close phylogenetic and geographic proximity to *E. globulus* (Duncan 2005). As such, these eucalypts were ideal for understanding how two closely related species are affected by limited water availability.

The specific aims of this research were to determine; 1) if water limitation affects *E. globulus* and *E. viminalis* PSM concentrations and if the response varies between levels of water availability, 2) if water limitation differentially affects PSM concentrations of juvenile leaves divided into within-plant foliage age classes. We predict that water limitation will decrease PSM concentrations due to limited photosynthetic carbon assimilation and within-plant transport of resources (Bréda *et al.* 2006). We also expect that leaf C:N will decrease due to constrained carbon assimilation and increased amino acid accumulation (Lawlor & Cornic 2002), and that young leaves will contain the greatest concentrations of many PSMs (Silvestre *et al.* 1997).

2.3 Materials and methods

2.3.1 Plant material

Eucalyptus globulus seed (Forestry Tasmania seed lot 100000325; 42.38°S, 146.60°E; 380 m asl; 1164 mm mean average rainfall) and *E. viminalis* seed (Forestry Tasmania seed lot 100002291; 41.75 °S, 146.88 °E; 400 m asl; 601 mm mean average rainfall) of unknown genetic diversity were germinated and grown for 1 month in a naturally lit glasshouse (10 hrs daylight [mean 1340 µmol m^{-2} s^{-1}], 76%

humidity). Uniform size (cotyledons and 1 leaf pair) seedlings (12 per species) were selected and transplanted into individual plastic pots (base 38 x 38 mm, top 50 x 50 mm, height 118 mm). Potting mix contained eight parts composted fine pine bark: three parts coarse river sand, and N:P:K [19: 2.6: 10] at 1 g /L potting mix. The pH was adjusted to approximately 6.0 with the addition of dolomite lime at 3 kg/m^{-3}. Seedlings were grown for 3 months, then re-potted into larger pots (base 115 mm diameter, top 138 mm diameter, height 169 mm) containing equivalent potting mix with the addition of fertiliser (5 g Osmocote® 3-4 month [N14:P6.1 :K11.6] per pot). Plants were moved to a controlled environment glasshouse where they were watered daily and randomised weekly. Natural daylight (full sunlight mean 1070 $\mu mol\ m^{-2}\ s^{-1}$) was supplemented with incandescent light to provide 16 hrs light/8 hrs dark at 18 ^{o}C/15 ^{o}C respectively, with 53% humidity. Chelated iron (1 ml/L water) was added once to all seedlings. Plants were grown for a further five weeks before experimental treatments were applied.

2.3.2 Experimental treatments

Four individuals of each species were allocated to a control group (watered to field capacity), four to a moderate water treatment (50% replacement of water required to reach field capacity), and the remaining four to a low water treatment (25% replacement of water required to reach field capacity). Each potted plant was initially watered to saturation, then left to drain for 15 min to remove excess water (field capacity). A capped section of PVC pipe (120 mm long, 40 mm diameter) was placed on the potting mix surface parallel with each stem. Each pot was wrapped in a plastic bag (Shvaleva *et al.* 2006) which was secured around the stem below the first leaf pair and the PVC pipe in order to prevent evaporation from the potting mix but to facilitate continued watering through the pipe. Pots were then weighed to provide total mass at field capacity.

At the beginning of the experiment, water was withheld from six month old plants of both species in both water limitation treatments (not including controls) until each plant was beginning to wilt. Once wilted, pots were re-weighed to establish pot weight at wilting, and replacement water mass calculated for each individual pot (Ma *et al.* 2010). Control plants were watered every 2 days, drought treatment plants every 3 days, with transpirational water loss measured gravimetrically. Control plants were not watered daily as the sealed pots retained sufficient water over a two day period to not require re-watering. Leaf water potential (Ψ_{leaf}) of each plant was measured on a single leaf using a Scholander pressure chamber (PMS, Albany, OR, USA) on the third day of the watering cycle prior to harvesting (Blackman *et al.* 2009) (treatment means = control -0.16 MPa; moderate water -0.33 MPa; low water -1.14 MPa). Due to these watering regimes, the moderate water plants showed signs of turgor loss, and the low water plants were heavily wilted every third day prior to re-watering. Control plants never wilted. All pots were randomised weekly within the glasshouse, and treatment duration was 3 months.

2.3.3 Sample collection

Leaf harvesting from all plants occurred at the end of the three month experimental period on day three of the watering cycle when pots contained the least water. Plant height was measured from the soil surface to the base of the apical bud. For each plant, leaves were divided into young (new growth, not fully expanded, developed during treatment), intermediate (fully formed juvenile foliage, developed before and during treatment) and old (tougher dark coloured juvenile leaves at base of stem and branches, developed prior to treatment) age groups based on leaf size, colour and location on plant (Close *et al.* 2005). Leaves in each leaf class were removed from the stem of a single plant and pooled (total of three sample bags per plant). A random sub-sample of leaves from each bag were frozen for essential oil analysis, and the remaining leaves were weighed, frozen (-12 °C), freeze dried, re-weighed, ground in a Cyclotec^{TM} 1093 cyclone mill (Foss, Hillerød, DK) and passed

through a 1 mm sieve for analysis of primary chemistry (Perkin Elmer 2400 Series II elemental analyser, Waltham, MA, USA), total phenolics (TPs), condensed tannins (CTs), sideroxylonal A and macrocarpal G (both FPCs). Sample mass prior to and post lyophilisation was used for quantification of fresh leaf water content (100-[dry mass/fresh mass x100]), expressed as percentage fresh weight (%FW) and referred to as 'leaf % water' hereafter.

2.3.4 Secondary chemistry

Extraction and analysis of essential oil follows the method outlined by McKiernan *et al.* (2012). Briefly, oil was extracted from frozen leaf samples in a dichloromethane (DCM) stock solution containing n-heptadecane as an internal standard. The extraction procedure was carried out for all 36 *E. globulus* samples (twelve plants × three age classes per plant) and 36 *E. viminalis* samples (twelve plants × three age classes per plant). Essential oil components (fourteen mono- and seven sesquiterpenes) were quantified using a Varian 450-Gas Chromatograph coupled to a Flame Ionization Detector with nitrogen as carrier gas, using Varian Galaxie software. Selected samples were also analysed by combined GC-MS on a Varian 3800 GC coupled to a Bruker 300-MS triple quadrupole mass spectrometer to assist in compound identification. Twenty-one individual oil components were positively identified through a combination of the NIST Mass Spectral database (NIST/EPA/NIH mass spectral library, NIST 08, August 2008, US Department of Commerce), a comprehensive in-house database of mass spectra of essential oil components, and Kovats' retention indices (Davies 1990). Other low concentration compounds were unidentifiable. Concentrations of 1,8-cineole and α-pinene were expressed as mg g^{-1} DM (dry matter) using standards, while total oil yield and other oil components were expressed as mg g^{-1} DM cineole equivalents.

Phenolics were extracted from all freeze-dried and ground leaf samples (Hagerman 2002) and quantified in duplicate using the modified Prussian Blue assay (Graham 1992). Absorbance was read at 700 nm using a UV-visible spectrophotometer (Varian Cary 1E, CA, USA), quantified using a range of 0.004 M gallic acid standards (Sigma G-7384), and results were expressed as mg g^{-1} DM gallic acid equivalents. Phenolic extracts were also used to quantify CTs in duplicate using the method described by Porter *et al*. (1986). Condensed tannin absorbance was read at 550 nm and quantified using a sorghum tannin standard (Hagerman 2002). Results were expressed as mg g^{-1} DM sorghum tannin equivalents. We present and discuss TP and CT concentrations as estimates defined by the method used, expressed in terms of the standard equivalents, with no characterisation of the compounds. Therefore, the actual phenols assayed using the modified Prussian Blue method (Graham 1992) and acid butanol method (Porter *et al.* 1986) are unknown in either species, and the actual concentrations may differ from the concentration using other analytical methods. These methods are appropriate to quantify the gross phenol concentrations and the treatment impacts on these concentrations.

We assayed two FPCs (sideroxylonal A and macrocarpal G) by High Performance Liquid Chromatography (HPLC) following methods of Wallis and Foley (2005). Sideroxylonal A was expressed as mg g^{-1} DM, while macrocarpal G was expressed as mg g^{-1} DM equivalents of macrocarpal A, using standards described by Eyles *et al.* (2003a).

2.3.5 Statistical analysis

All analyses were completed using SAS statistical software package (version 9.2, SAS Institute Inc., Cary USA). Species, treatment and interaction fixed effects were s for each dependant variable (except plant height) using a mixed model (PROC MIXED with foliage age class treated as a repeated measure within each individual

plant [random]). Height data were analysed by general linear model (PROC GLM) fitting species, treatment and interaction fixed effects. Residuals for all variables were checked for assumptions of normality and heterogeneity of variances, and transformations were made where necessary. All traits were transformed to their natural logarithm except plant height, leaf % water, TPs, carbon, nitrogen and C:N. Due to the number of individual analyses performed, the false discovery rate was controlled following Benjamini and Hochberg (2000) using the associated supplemental file. In order to summarise the multivariate patterns of chemical variation, a number of chemical traits were analysed using discriminant analysis (PROC DISCRIM) with each species x treatment x foliage age class combination treated as a separate group.

2.4 Results

2.4.1 Plant growth

Juvenile *E. globulus* were 8.5% taller than *E. viminalis* plants ($F_{1,23}$=6.98; *P*=0.02). There was a significant treatment effect on plant height ($F_{2,23}$=7.93; *P*=0.003), where height was reduced by 14.4% in the low water treatment (*P*=0.003) and by 10.7% in the moderate water treatment (*P*=0.03) compared to controls. There was no species × treatment interaction influencing plant height ($F_{2,23}$=0.22; *P*=0.81).

2.4.2 Leaf % water

A significant treatment x foliage age class interaction indicated that water limitation affected leaf % water of foliage age classes differently (Table 1). Specifically, leaf % water of young leaves decreased in both reduced water treatments, while the leaf % water of intermediate age leaves decreased only in the low water treatment (Figure 1a). The leaf % water of old leaves did not differ between treatments (Figure 1a). A species × foliage age class interaction was also

revealed where old *E. globulus* leaves contained proportionally more water than both young (3%) and intermediate (4%) leaves (Table 1). In contrast, young and old *E. viminalis* leaves contained proportionally more water than did intermediate age *E. viminalis* leaves (2% and 1.5% respectively). There was no three-way interaction affecting leaf % water.

Table 1. Results of mixed model analyses for variation of plant secondary metabolite (PSM) concentrations in juvenile *Eucalyptus globulus* and *E. viminalis* leaves between species, water treatments (control, moderate water, low water), foliage ages (young, intermediate, old), and all interactions[#]

Trait	Species		Treatment		Foliage Age Class		Species x Treatment		Species x Foliage Age Class		Treatment x Foliage Age Class		Species x Treatment x Foliage Age Class	
	$F_{1,18}$	*P*	$F_{2,18}$	*P*	$F_{2,18}$	*P*	$F_{2,18}$	*P*	$F_{2,18}$	*P*	$F_{4,18}$	*P*	$F_{4,18}$	*P*
Total oil	74	***	2		142	***	2		6	**	2		1	
1,8-Cineole	29	***	2		100	***	1		13	**	1		1	
Cymene	38	***	1		0		2		2		1		0	
Geraniol	9	**	1		91	***	1		5	*	6	**	2	
Limonene	30	***	2		99	***	0		17	***	2		1	
Methyl geranate	2		0		9	**	1		2		1		0	
Myrcene	58	***	3		133	***	2		16	***	4		1	
Neral	15	**	1		7	**	3		3		1		1	
Terpinen-4-ol	1		1		47	***	0		11	**	2		0	
Terpinyl acetate	9	**	4		42	***	0		26	***	4		3	
α-Phellandrene	2		2		46	***	1		2		0		0	
α-Pinene	68	***	7		177	***	7		7	**	1		0	
α-Terpineol	3		0		79	***	0		1		2		0	
β-Pinene	138	***	2		147	***	3		4	*	3		1	
γ-Terpinene	3		0		51	***	0		3		1		1	
Alloaromadendrene[a]	229	***	4		84	***	3		2		1		0	
Aromadendrene[a]	189	***	3		67	***	3		1		2		0	
Bicyclogermacrene[a]	173	***	2		75	***	0		4	*	6	**	2	
Globulol[a]	200	***	4		72	***	4		2		2		0	
Rosifoliol[a]	173	***	2		19	***	2		5	*	4		1	
Viridiflorol[a]	190	***	2		31	***	1		15	**	3		4	
α-Gurjunene[a]	142	***	3		67	***	1		3		3		2	
Total phenolics[b]	24	**	10	**	6	**	0		3		3		3	
Condensed tannins[b]	19	**	1		71	***	1		1		2		2	
Macrocarpal G[bc]	236	***	3		90	***	1		10	**	4		2	
Sideroxylonal A[bc]	4		2		50	***	2		15	**	1		1	
Leaf % water [d]	1		3		55	***	1		8	**	11	***	2	
Carbon[e]	12	**	2		2		7		1		4		2	
Nitrogen[e]	9	**	6		10	**	0		3		1		2	
C:N[f]	8	**	9	**	10	**	2		4	*	2		2	

[#] Significance after False Discovery Rate (FDR) control following Benjamini and Hochberg (2000), *indicate $P \leq 0.05$, **indicate $P < 0.01$, *** indicate $P < 0.001$. Italics indicate monoterpene, [a] indicates sesquiterpene, [b] indicates phenolic compound, [c] indicates formylated phloroglucinol compound (FPC), [d] water content of fresh leaves expressed as the proportion of fresh leaf mass, [e] indicates primary chemical. Variable 'foliage' included in mixed analyses as repeated measures within each plant.

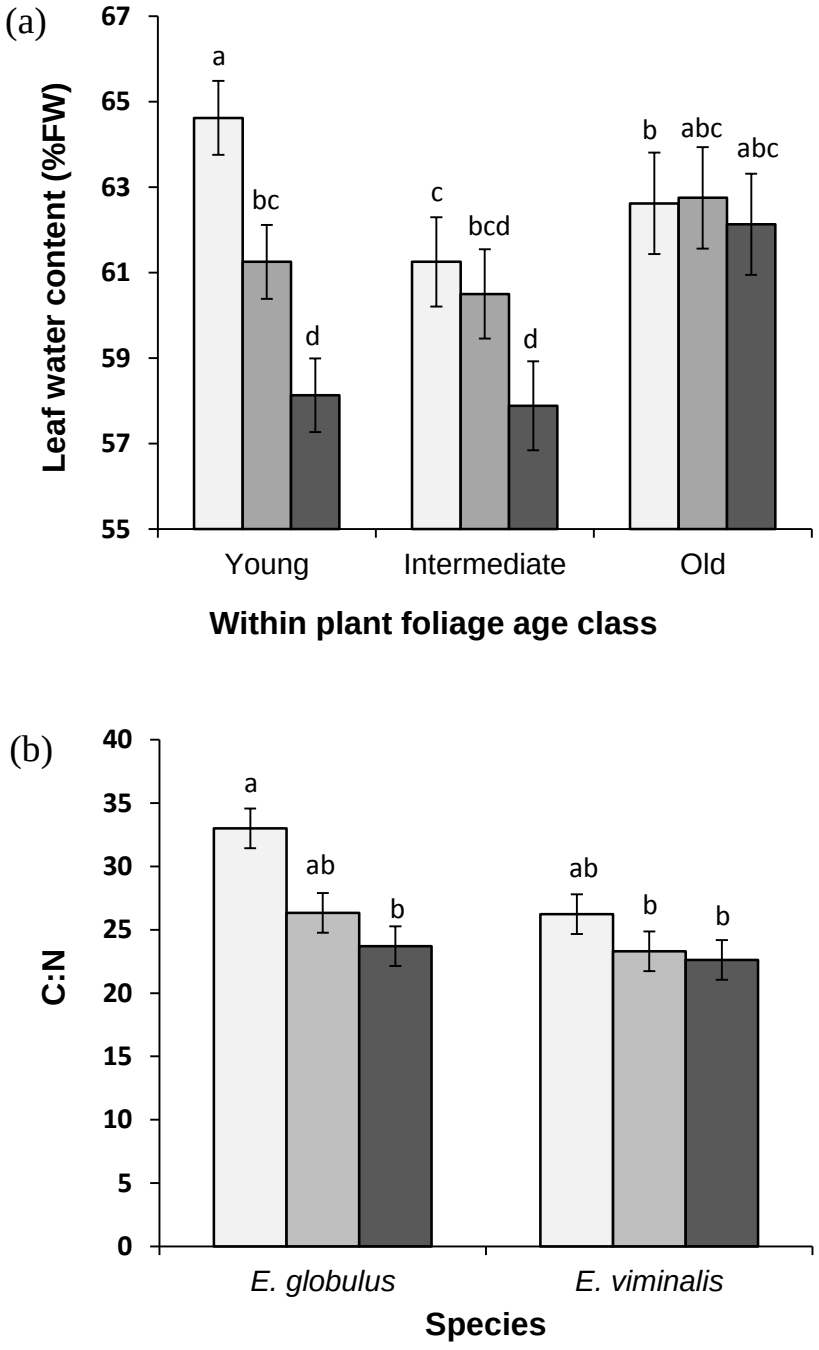

Figure 1. Mean (±SE) leaf % water (a) and leaf carbon to nitrogen (C:N) ratio (b) in juvenile *Eucalyptus globulus* and *E. viminalis* grown under control (field capacity, light bars), moderate water (grey bars) and low water (dark bars) treatments. Leaf water content expressed as proportion of fresh leaf mass (% FW). Letters indicate significant difference ($P \leq 0.05$) after Tukey's post-hoc test between all columns within (a) or within (b). Data did not require transformation to meet model assumptions.

2.4.3 Primary chemistry

Leaf carbon concentration varied between species (Table 1), where carbon made up 1.5% more of dry *E. viminalis* leaves than *E. globulus* leaves (Table 2). There were no significant treatment, foliage age class or interaction effects on leaf carbon (Table 1). Leaf nitrogen concentration varied between species and between foliage age classes (Table 1). Specifically, nitrogen made up 0.33% more of *E. viminalis* dry leaf mass than in *E. globulus* leaves, and nitrogen was highest in young leaves (Table 3). Leaf nitrogen was not altered by water limitation and there were no interactions (Table 1). Overall, the ratio of C:N decreased by 16% under moderate water and by 22% under low water availability (Table 1). While no species × treatment interaction affected leaf C:N (Table 1), post-hoc tests revealed that the effect of reduced water on *E. viminalis* C:N was non-significant (Figure 1b) even though the trend was consistent between species. The C:N ratio differed between foliage age classes of *E. globulus* only (Table 3), explaining a species × foliage age class interaction (Table 1) where the ratio of C:N in old *E. globulus* leaves was 16% higher than in young leaves (Table 3).

Table 2. Least squares means (±SE) of traits from juvenile *Eucalyptus globulus* and *E. viminalis* grown in three water availability (drought) treatments[§]

	E. globulus			*E. viminalis*		
Trait	**Control**	**Moderate water**	**Low water**	**Control**	**Moderate water**	**Low water**
Total oil[1]	63.46 (5.17)[a]	62.09 (5.17)[a]	52.66 (5.17)[a]	24.17 (5.17)[b]	35.41 (5.17)[b]	26.61(5.17)[b]
1,8-Cineole[2]	32.95 (3.44)[a]	34.82 (3.44)[a]	27.58 (3.44)[ac]	15.26 (3.44)[b]	23.21 (3.44)[ab]	17.88 (3.44)[bc]
Alloaromadendrene[1]	0.79 (0.05)[a]	0.72 (0.05)[a]	0.71 (0.05)[a]	0.11 (0.05)[b]	0.09 (0.05)[bc]	0.04 (0.05)[c]
Aromadendrene[1]	2.21 (0.20)[a]	1.82 (0.20)[a]	2.04 (0.20)[a]	0.21 (0.20)[b]	0.17 (0.20)[bc]	0.07 (0.20)[c]
Bicyclogermacrene[1]	0.14 (0.02)[a]	0.16 (0.02)[a]	0.10 (0.02)[a]	0.02 (0.02)[b]	0.02 (0.02)[b]	0.01 (0.02)[b]
Cymene[1]	0.04 (0.04)[a]	0.04 (0.04)[a]	0.04 (0.04)[a]	0.27 (0.04)[b]	0.10 (0.04)[ab]	0.14 (0.04)[a]
Geraniol[1]	0.03 (0.02)[a]	0.02 (0.02)[a]	0.02 (0.02)[a]	0.07 (0.02)[a]	0.10 (0.02)[a]	0.04 (0.02)[a]
Globulol[1]	1.49 (0.12)[a]	1.20 (0.12)[a]	1.34 (0.12)[a]	0.19 (0.12)[b]	0.16 (0.12)[bc]	0.05 (0.12)[c]
Limonene[1]	2.79 (0.30)[a]	3.17 (0.30)[a]	2.53 (0.30)[a]	1.44 (0.30)[b]	2.06 (0.30)[ab]	1.50 (0.30)[b]
Methyl geranate[1]	0.02 (0.01)[a]	0.02 (0.01)[a]	0.01 (0.01)[a]	0.02 (0.01)[a]	0.04 (0.01)[a]	0.03 (0.01)[a]
Myrcene[1]	0.43 (0.04)[a]	0.43 (0.04)[a]	0.35 (0.04)[a]	0.16 (0.04)[b]	0.19 (0.04)[b]	0.11 (0.04)[b]
Neral[1]	0.04 (0.01)[ab]	0.02 (0.01)[a]	0.01 (0.01)[a]	0.04 (0.01)[ab]	0.07 (0.01)[b]	0.04 (0.01)[ab]
Rosifoliol[1]	0.29 (0.03)[a]	0.27 (0.03)[a]	0.28 (0.03)[a]	0.04 (0.03)[b]	0.04 (0.03)[b]	0.02 (0.03)[b]
Terpinen-4-ol[1]	0.11 (0.02)[a]	0.11 (0.02)[a]	0.10 (0.02)[a]	0.13 (0.02)[a]	0.10 (0.02)[a]	0.07 (0.02)[a]
Terpinyl acetate[1]	2.68 (0.46)[a]	1.29 (0.46)[ab]	1.05 (0.46)[ab]	0.32 (0.46)[ab]	0.01 (0.46)[b]	0.02 (0.46)[b]
Viridiflorol[1]	0.59 (0.05)[a]	0.48 (0.05)[a]	0.52 (0.05)[a]	0.08 (0.05)[b]	0.07 (0.05)[b]	0.04 (0.05)[b]
α-Gurjunene[1]	0.72 (0.05)[a]	0.60 (0.05)[a]	0.55 (0.05)[a]	0.09 (0.05)[b]	0.06 (0.05)[b]	0.02 (0.05)[b]
α-Phellandrene[1]	0.02 (0.05)[a]	0.02 (0.05)[a]	0.01 (0.05)[a]	0.18 (0.05)[a]	0.03 (0.05)[a]	0.02 (0.05)[a]
α-Pinene[1]	9.81 (1.02)[a]	10.35 (1.02)[a]	8.91 (1.02)[a]	1.52 (1.02)[b]	5.27 (1.02)[ac]	3.28 (1.02)[c]
α-Terpineol[1]	1.05 (0.23)[a]	0.88 (0.23)[a]	0.90 (0.23)[a]	1.19 (0.23)[a]	1.29 (0.23)[a]	1.20 (0.23)[a]
β-Pinene[1]	0.43 (0.03)[a]	0.40 (0.03)[a]	0.36 (0.03)[a]	0.09 (0.03)[b]	0.16 (0.03)[b]	0.09 (0.03)[b]
γ-Terpinene[1]	0.14 (0.36)[a]	0.11 (0.36)[a]	0.13 (0.36)[a]	0.95 (0.36)[a]	0.37 (0.36)[a]	0.43 (0.36)[a]
Total phenolics[3]	149.62 (6.37)[a]	122.38 (6.37)[ab]	120.65 (6.37)[b]	118.72 (6.37)[b]	100.77 (6.37)[b]	96.40 (6.37)[b]
Condensed tannins[4]	10.66 (2.36)[ab]	6.78 (2.36)[a]	6.11 (2.36)[ac]	16.33 (2.36)[bc]	12.01 (2.36)[ab]	16.22 (2.36)[b]
Sideroxylonal A[2]	5.70 (1.24)[a]	6.77 (1.24)[a]	3.87 (1.24)[a]	5.36 (1.24)[a]	9.49 (1.24)[a]	8.05 (1.24)[a]
Macrocarpal G[5]	5.78 (0.30)[a]	4.71 (0.30)[a]	4.28 (0.30)[a]	0.88 (0.30)[b]	0.71 (0.30)[b]	0.47 (0.30)[b]
Carbon[6]	44.29 (0.56)[a]	45.25 (0.56)[ab]	43.33 (0.56)[a]	44.54 (0.56)[a]	45.62 (0.56)[ab]	47.36 (0.56)[b]
Nitrogen[6]	1.37 (0.13)[a]	1.76 (0.13)[ab]	1.84 (0.13)[ab]	1.75 (0.13)[ab]	2.00 (0.13)[b]	2.19 (0.13)[b]
C:N ratio	33.00 (1.57)[a]	26.33 (1.57)[ab]	23.71 (1.57)[b]	26.24 (1.57)[ab]	23.30 (1.57)[b]	22.62 (1.57)[b]
Height [7]	885.00 (32.45)[a]	813.50 (32.45)[ab]	774.50 (32.45)[ab]	838.25 (32.45)[ab]	724.75 (32.45)[b]	700.00 (32.45)[b]
Leaf % water[8]	0.62 (0.01)[a]	0.62 (0.01)[a]	0.60 (0.01)[a]	0.64 (0.01)[a]	0.61 (0.01)[a]	0.59 (0.01)[a]

[§1] results expressed as $mg.g^{-1}$.DM cineole equivalents, [2] results expressed as $mg.g^{-1}$.DM, [3] results expressed as $mg.g^{-1}$.DM gallic acid equivalents, [4] results expressed as $mg.g^{-1}$.DM sorghum tannin equivalents, [5] results expressed as $mg.g^{-1}$.DM macrocarpal A equivalents, [6] results expressed as percentage dry leaf mass, [7] results expressed in mm, [8] results expressed as the proportion of fresh leaf mass. Letters indicate significance ($P \leq 0.05$ with no false discovery rate control) after Tukey's post-hoc test between treatments and species for each trait across the six columns. Data for all traits were transformed to their natural logarithm except height, leaf % water, total phenolics, carbon, nitrogen and C:N.

Table 3. Least squares means (±SE) of traits from juvenile *Eucalyptus globulus* and *E. viminalis* leaves grouped into three within-plant leaf age classes ˆ

	E. globulus			*E. viminalis*		
Trait	**Young**	**Intermediate**	**Old**	**Young**	**Intermediate**	**Old**
Total oil[1]	74.18 (3.85)^{a}	64.13 (3.60)^{b}	39.89 (2.83)^{c}	41.11 (3.85)^{c}	28.58 (3.60)^{d}	16.50 (2.83)^{e}
1,8-Cineole[2]	36.93 (2.58)^{a}	34.91 (2.25)^{a}	23.52 (1.73)^{bc}	26.23 (2.58)^{b}	18.93 (2.25)^{c}	11.19 (1.73)^{d}
Alloaromadendrene[1]	1.04 (0.04)^{a}	0.80 (0.05)^{b}	0.38 (0.03)^{c}	0.12 (0.04)^{d}	0.08 (0.05)^{e}	0.04 (0.03)^{f}
Aromadendrene[1]	2.93 (0.15)^{a}	2.15 (0.16)^{b}	1.00 (0.09)^{c}	0.23 (0.15)^{d}	0.15 (0.16)^{e}	0.07 (0.09)^{f}
Bicyclogermacrene[1]	0.20 (0.01)^{a}	0.14 (0.02)^{a}	0.06 (0.01)^{b}	0.03 (0.01)^{c}	0.02 (0.02)^{cd}	0.01 (0.01)^{d}
Cymene[1]	0.03 (0.03)^{a}	0.04 (0.03)^{a}	0.04 (0.02)^{a}	0.19 (0.03)^{b}	0.18 (0.03)^{b}	0.15 (0.02)^{b}
Geraniol[1]	0.04 (0.02)^{ae}	0.02 (0.01)^{b}	0.01 (0.003)^{c}	0.12 (0.02)^{de}	0.06 (0.01)^{a}	0.02 (0.003)^{bc}
Globulol[1]	1.87 (0.08)^{a}	1.45 (0.11)^{b}	0.70 (0.06)^{c}	0.20 (0.08)^{d}	0.14 (0.11)^{e}	0.08 (0.06)^{f}
Limonene[1]	3.30 (0.24)^{a}	3.16 (0.20)^{ac}	2.06 (0.16)^{bf}	2.49 (0.24)^{cf}	1.64 (0.20)^{bd}	0.87 (0.16)^{e}
Methyl geranate[1]	0.02 (0.01)^{ab}	0.02 (0.01)^{ab}	0.01 (0.01)^{ab}	0.05 (0.01)^{ab}	0.03 (0.01)^{a}	0.01 (0.01)^{b}
Myrcene[1]	0.56 (0.04)^{a}	0.46 (0.02)^{a}	0.20 (0.02)^{bc}	0.28 (0.04)^{b}	0.13 (0.02)^{c}	0.04 (0.02)^{d}
Neral[1]	0.03 (0.01)^{ac}	0.02 (0.01)^{a}	0.02 (0.01)^{a}	0.08 (0.01)^{b}	0.05 (0.01)^{bc}	0.02 (0.01)^{a}
Rosifoliol[1]	0.35 (0.02)^{a}	0.32 (0.02)^{a}	0.17 (0.01)^{b}	0.04 (0.02)^{c}	0.03 (0.02)^{c}	0.02 (0.01)^{c}
Terpinen-4-ol[1]	0.08 (0.02)^{ac}	0.13 (0.02)^{b}	0.11 (0.01)^{b}	0.11 (0.02)^{ab}	0.13 (0.02)^{bc}	0.07 (0.01)^{a}
Terpinyl acetate[1]	1.59 (0.25)^{ab}	1.91 (0.29)^{a}	1.52 (0.28)^{b}	0.17 (0.25)^{abc}	0.13 (0.29)^{d}	0.06 (0.28)^{e}
Viridiflorol[1]	0.70 (0.03)^{a}	0.59 (0.04)^{a}	0.30 (0.02)^{b}	0.07 (0.03)^{c}	0.07 (0.04)^{c}	0.05 (0.02)^{c}
α-Gurjunene[1]	1.06 (0.07)^{a}	0.64 (0.06)^{b}	0.17 (0.02)^{c}	0.10 (0.07)^{c}	0.05 (0.06)^{d}	0.02 (0.02)^{d}
α-Phellandrene[1]	0.03 (0.05)^{ad}	0.02 (0.03)^{ab}	0.01 (0.01)^{b}	0.13 (0.05)^{ac}	0.08 (0.03)^{c}	0.02 (0.01)^{bd}
α-Pinene[1]	13.49 (0.86)^{a}	9.84 (0.67)^{b}	5.73 (0.46)^{c}	5.50 (0.86)^{c}	3.04 (0.67)^{d}	1.54 (0.46)^{e}
α-Terpineol[1]	1.18 (0.21)^{acde}	1.08 (0.14)^{acde}	0.57 (0.07)^{b}	1.70 (0.21)^{c}	1.28 (0.14)^{d}	0.70 (0.07)^{bd}
β-Pinene[1]	0.54 (0.02)^{a}	0.41 (0.01)^{b}	0.23 (0.02)^{c}	0.18 (0.02)^{c}	0.11 (0.01)^{d}	0.05 (0.02)^{e}
γ-Terpinene[1]	0.17 (0.39)^{acde}	0.13 (0.17)^{acde}	0.08 (0.07)^{bf}	1.03 (0.39)^{c}	0.49 (0.17)^{d}	0.22 (0.07)^{ef}
Total phenolics[3]	135.02 (5.56)^{a}	124.01 (4.31)^{a}	133.62 (5.21)^{a}	118.15 (5.56)^{ab}	101.95 (4.31)^{b}	95.79 (5.21)^{b}
Condensed tannins[4]	3.75 (1.02)^{a}	6.46 (1.24)^{bd}	13.33 (2.30)^{ce}	8.92 (1.02)^{dc}	13.42 (1.24)^{e}	22.21 (2.30)^{f}
Sideroxylonal A[2]	6.33 (0.97)^{a}	5.31 (0.71)^{ab}	4.70 (0.59)^{b}	10.48 (0.97)^{c}	7.78 (0.71)^{a}	4.66 (0.59)^{ab}
Macrocarpal G[5]	7.57 (0.32)^{a}	4.54 (0.15)^{b}	2.66 (0.15)^{c}	0.92 (0.32)^{d}	0.60 (0.15)^{e}	0.54 (0.15)^{e}
Carbon[6]	45.1 (0.42)^{ab}	44.47 (0.41)^{ab}	43.29 (0.63)^{b}	46.05 (0.42)^{a}	46.02 (0.41)^{a}	45.45 (0.63)^{ab}
Nitrogen[6]	1.83 (0.08)^{ac}	1.61 (0.09)^{abd}	1.52 (0.10)^{b}	2.02 (0.08)^{c}	1.99 (0.09)^{cd}	1.92 (0.10)^{abc}
C:N	25.06 (0.94)^{a}	28.23 (1.19)^{bc}	29.75 (1.20)^{b}	23.42 (0.94)^{a}	24.17 (1.19)^{ac}	24.57 (1.20)^{ab}
Leaf % water[7]	0.61 (0.01)^{ac}	0.60 (0.01)^{ac}	0.64 (0.01)^{bd}	0.62 (0.01)^{cd}	0.60 (0.01)^{ab}	0.61 (0.01)^{cd}

ˆ[1] results expressed as mg.g^{-1}.DM cineole equivalents, [2] results expressed as mg.g^{-1}.DM, [3] results expressed as mg.g^{-1}.DM gallic acid equivalents, [4] results expressed as mg.g^{-1}.DM sorghum tannin equivalents, [5] results expressed as mg.g^{-1}.DM Macrocarpal A equivalents, [6] results expressed as percentage dry leaf, [7] results expressed as the proportion of fresh leaf mass. Letters indicate significance ($P \leq 0.05$ with no false discovery rate control) after Tukey's post-hoc test between foliage age class and species for each trait across the six columns. Data for all traits were transformed to their natural logarithm except height, leaf % water, total phenolics, carbon, nitrogen and C:N.

2.4.4 Essential oil

Most oil components (full list in Table 1) varied quantitatively between species and between foliage age classes, whereby *E. globulus* contained higher concentrations of most individual oil components, and young leaves contained higher concentrations than older leaves of the same plants (Tables 1-3). However, the total oil yield and concentrations of eleven oil components exhibited significant species × foliage age class interactions (Figure 2a; Table 1). Water limitation did not affect the total oil yield, or concentrations of most oil components (mono- or sesquiterpene) at the whole plant level (Table 1). However, variation in concentrations of geraniol and bicyclogermacrene resulted from differing treatment × foliage age class interactions (Table 1). Firstly, concentrations of geraniol in intermediate age foliage of low water plants were nearly half that of control leaves of the same age class (P=0.02), but geraniol concentrations in young and old leaves were unaffected. In contrast, concentrations of bicyclogermacrene in young leaves decreased by 28% under the low water treatment (P=0.008), yet remained stable in intermediate and old leaves. No species × treatment or 3-way interaction was found to affect the concentrations of total oil yield or any oil component (Table 1).

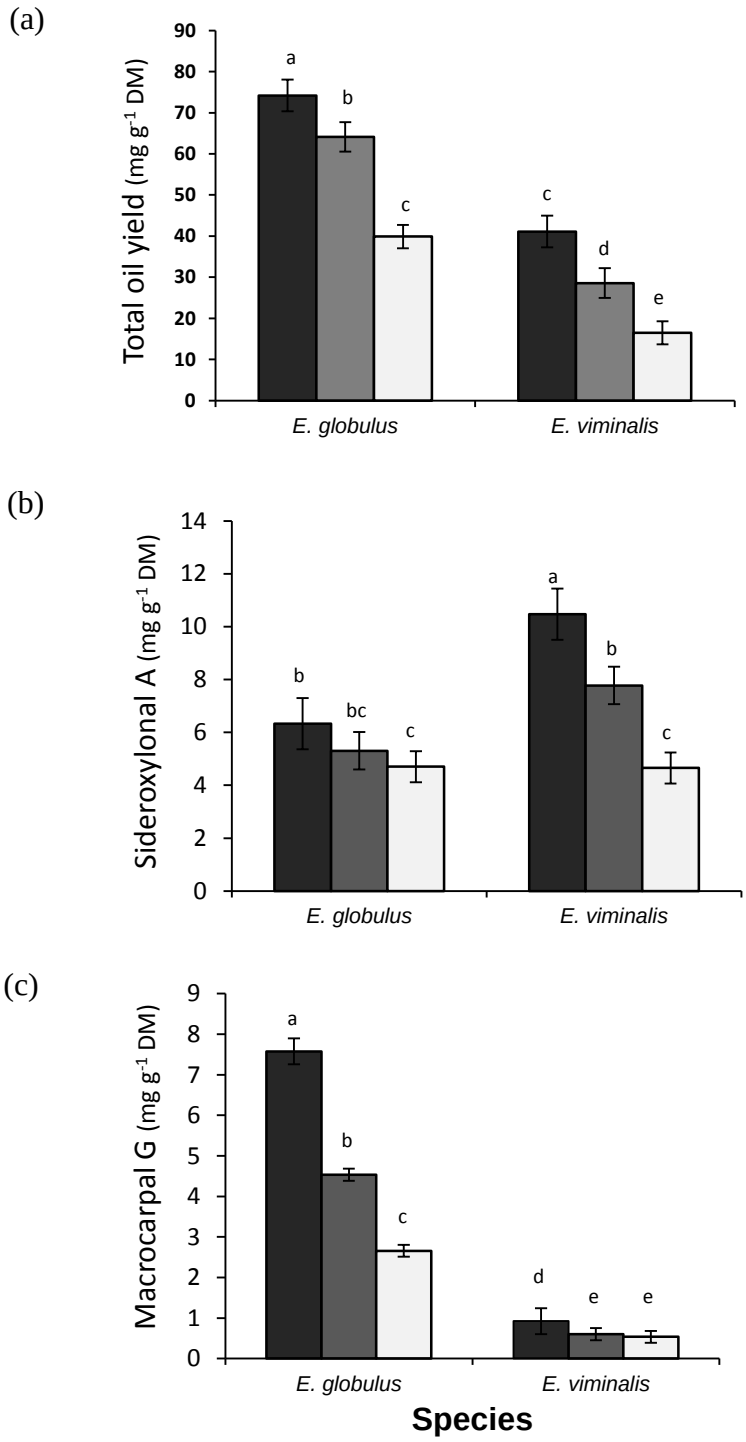

Figure 2. Mean (±SE) within-plant concentrations of total oil (a) sideroxylonal A (b) and macrocarpal G (c) of young (black bars), intermediate (grey bars) and older (light bars) foliage age classes within *E. globulus* and *E. viminalis* plants. Letters indicate significant difference ($P \leq 0.05$) of log transformed data after Tukey's post-hoc test between all columns within (a), (b) or (c). Total oil expressed as mg g^{-1} DM cineole equivalents and Macrocarpal G expressed as mg g^{-1} DM macrocarpal A equivalents.

2.4.5 Formylated phloroglucinol compounds (FPCs)

Both sideroxylonal A and macrocarpal G concentrations were affected by species × foliage age class interactions (Table 1). Young *E. viminalis* leaves contained the highest sideroxylonal A concentration at a mean of 10.5 mg g^{-1} DM (Figure 2b). Young leaves of *E. globulus* contained higher concentrations than older leaves (25%), but young *E. viminalis* leaves contained 55% more sideroxylonal A than old leaves (Figure 2b). *Eucalyptus globulus* leaves contained seven times more macrocarpal G than *E. viminalis* leaves (Table 3). The same leaf age trend was found for macrocarpal G concentrations as was found for sideroxylonal A, where young *E. globulus* leaves had nearly three times as much macrocarpal G as old leaves, and young *E. viminalis* had 40% more than old leaves of that species (Figure 2c). The drought treatments had no statistically significant effect on sideroxylonal A or macrocarpal G concentrations in either species (Table 1).

2.4.6 Total phenolics and condensed tannin

Eucalyptus globulus contained 20% higher TPs, and almost half the CT concentrations, than did *E. viminalis* (Table 1, 2). Total phenolic concentrations in both species were reduced by the water treatments (Table 1), with leaves from plants in the moderate water treatment containing 17% less TPs than controls, and leaves of low water plants containing 19% less than the controls (Figure 3). Concentrations of CTs were not affected by drought treatments (Table 1). Within plants, TP concentrations varied between foliage age classes (Table 1), where young leaves contained 11% higher concentrations than intermediate age leaves and 9% more than old leaves (Table 3). In contrast, young leaves contained much lower concentrations of CTs than intermediate (36%) and old leaves (64%), and intermediate leaves 44% less than old leaves (Table 3).

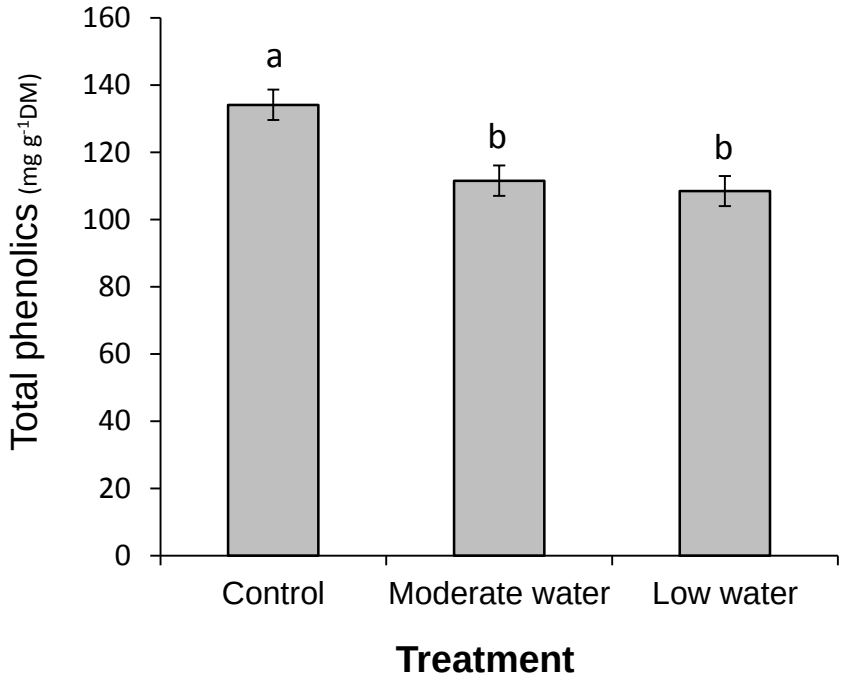

Figure 3. Mean (±SE) concentration of total phenolics in juvenile *Eucalyptus* leaves under three watering treatments. Total phenolics expressed as mg g^{-1} DM gallic acid equivalents. Letters indicate significant differences ($P \leq 0.05$) after Tukey's post-hoc tests between all columns. Data did not require transformation to meet model assumptions.

2.4.7 Discriminant analysis and ordination using multiple chemical traits

The multivariate summary investigated differences between foliage age classes of each species grown under each watering treatment, and used sideroxylonal A, macrocarpal G, total phenolic, 1,8-cineole, α-pinene, β-pinene, myrcene, cymene, limonene, γ-terpinene, terpinen-4-ol, α-terpineol, neral, geraniol, α-gurjunene, aromadendrene, alloaromadendrene, globulol, viridiflorol and rosifoliol data. The resulting multivariate plot showed that the main difference in the overall PSM profile of leaves was between species along axis cv1 (explaining 71% of the total variation), then to a lesser extent between, foliage age classes (cv1 and cv2) within each species (Figure 4). In the two dimensional space the treatments had the least impact on overall chemical profile, but appear to be larger and more consistent within *E. viminalis* foliage compared to *E. globulus* (Figure 4). In summary, the degree of overall PSM variation resulting from the applied watering treatments (environment) was much less than the degree of variation observed between leaf age classes (genetic), and between species (genetic).

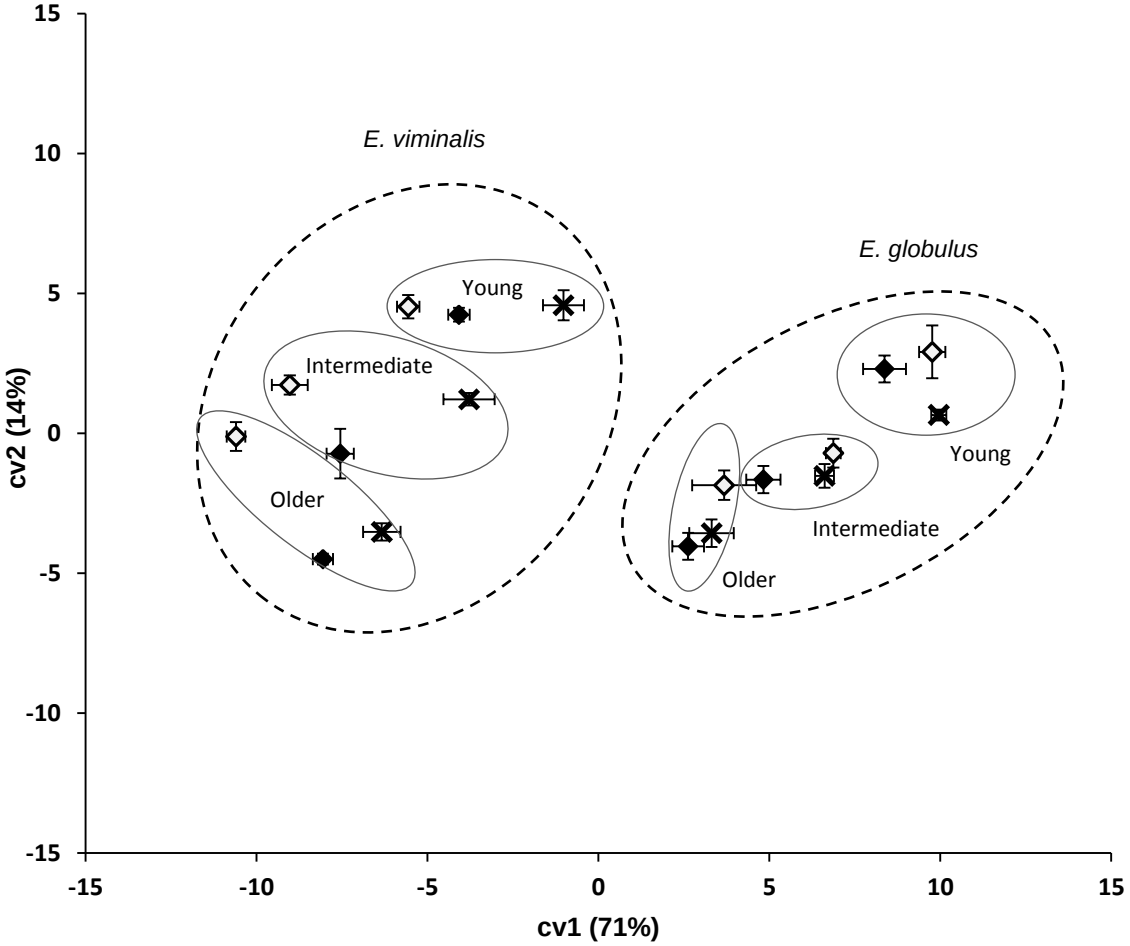

Figure 4. Ordination summarising the overall response of leaf chemical traits to water limitation between within-plant foliage age classes in juvenile *Eucalyptus globulus* and *E. viminalis*. Broken lines encircle species grouping, solid lines encircle foliage age class grouping within each species. Open diamonds indicate control, crosses indicate moderate water availability, closed diamonds indicate low water availability. Bars indicate standard errors. Percentages indicate the proportion of variation explained by each canonical coefficient. The axes are the first two canonical variates derived from discriminant analysis. The analysis was based on sideroxylonal A, macrocarpal G, total phenolic, 1,8-cineole, α-pinene, β-pinene, myrcene, cymene, limonene, γ-terpinene, terpinen-4-ol, α-terpineol, neral, geraniol, α-gurjunene, aromadendrene, alloaromadendrene, globulol, viridiflorol and rosifoliol data transformed to their natural logarithm, except total phenolics which did not require transformation.

2.5 Discussion

2.5.1 Effects of water limitation on whole plant PSM concentrations

This study highlights the quantitative variation of selected PSMs between two *Eucalyptus* species of section *Maidenaria*. Overall, juvenile *E. globulus* plants in our study contained higher concentrations of the assayed PSMs than *E. viminalis*. In contrast to the range of genetic-based PSM variation between these closely related species, plasticity of PSM expression during limited water availability was minimal in both species. Plants in the low and moderate water treatments regularly displayed symptoms of turgor loss, had greater vascular tension, and exhibited reduced growth and leaf C:N. Yet surprisingly, the expression of most PSMs remained stable, even in the young leaves which developed completely while water availability was limited. Water limitation usually leads to a reduction in plant above-ground growth (Chaves *et al.* 2003), however, these plants may also have prioritised the maintenance of leaf PSM concentrations over growth (Yang *et al.* 2012), or else growth was limited more than PSM biosynthesis by the level of reduced water availability (Herms & Mattson 1992). While we can only speculate about how trade-offs and resource prioritisation in these plants are influencing growth and differentiation, we can say that the total oil yield, concentrations of individual terpenes, CTs and the FPCs were unaffected by the reduced availability of water. In other systems, concentrations of these PSMs show variable responses to reduced water availability, with some species showing clear but often opposite effects (Doran & Bell 1994; Leicach *et al.* 2010) while others, like in our study, show a minimal response (Stone & Bacon 1994; Gleadow & Woodrow 2002; King *et al.* 2004; Pizarro & Bisigato 2010). These variable patterns could be explained by genetic or experimental variation, and perhaps in some systems the effects are evident only when water availability interacts with other environmental treatments such as nutrient availability (Doran & Bell 1994; Brooker 2000; Nik *et al.* 2008; Said-Al Ahl & Abdou 2009; Razmjoo *et al.* 2013).

The group of compounds that did show a response in our study was the TPs, which decreased in both *E. globulus* and *E. viminalis* leaves as a result of water limitation. The reason why reduced water availability only affected TP concentrations and not terpenes or FPCs is unclear, but may reflect differences in biosynthetic pathways between PSM classes (Dudareva *et al.* 2004; Ashour *et al.* 2010; Petersen *et al.* 2010; Wink 2010) or different within-leaf storage locations among PSM classes (Fahn 1979; Kutchan 2005). In fact, the stability of CT concentrations quantified using the acid butanol method indicates that the effect of reduced water availability is limited to a phenolic class/es not specifically quantified in this study, or a particular biosynthetic precursor not involved in CT synthesis. While we were unable to quantify other phenolic classes, candidates could include hydrolysable tannins (ellagitannis and/or gallotannins which are extracted along with the CTs) (Hagerman 1988), flavanoids, or any number of other compounds (e.g. caffeic acid).

Previous experiments examining the effects of reduced water availability on TP concentrations in eucalypts are few. However, in contrast to the results presented here, Gleadow and Woodrow (2002) found that TP concentrations of *E. cladocalyx* foliage were unaffected by water stress. The inconsistency between theirs and our findings may result from differences in treatments, plant species, phenolic compounds, or an interaction of these factors. Mixed patterns in phenolic responses to reduced water are not uncommon between genera (Ballhorn *et al.* 2011; Bettaieb *et al.* 2011; Gutbrodt *et al.* 2012a; Zhang *et al.* 2012), and effects vary even between cultivars within species (Sánchez-Rodríguez *et al.* 2012), however, many studies have demonstrated increasing phenolic concentrations due to limited water (Pizarro & Bisigato 2010; Azhar *et al.* 2011; Yadav *et al.* 2014). Ecologically, increased TP concentrations reduce mammal browsing, either directly (Wiggins *et al.* 2006b), or by reducing plant nitrogen availability (DeGabriel *et al.* 2009a), and increased TPs can also reduce larvae health and development (Walter *et al.* 2012). Therefore, it is likely that the decreases to TP concentrations in our leaves (in the order of 17-19%)

could increase plant palatability, especially with coinciding decreases in the C:N ratio.

2.5.2 Within-plant PSM variation between foliage age classes

Overall, young leaves contained higher concentrations of most PSMs compared to older leaves of the same juvenile plant, with the major exception being the opposing trend of CT concentrations. In fact, the variation in chemical concentrations between foliage age classes was greater than the level of variation resulting from the treatments (Figure 4). Similar variation was noted in another eucalypt species, *E. nitens*, (Close *et al.* 2005; Loney *et al.* 2006b) and has also been observed across other plant taxa including woody (Brunt *et al.* 2006; Estell *et al.* 2013; Rummukainen *et al.* 2013) and non-woody species (Gutbrodt *et al.* 2012b; Holeski *et al.* 2013). High PSM concentrations of newly developed juvenile leaves almost certainly reflects a strong ecological benefit to the plant, such as providing these leaves with increased defense against herbivory (Brunt *et al.* 2006; Loney *et al.* 2006b). Interestingly, many species of eucalypt exhibit clear heteroblastic leaf phase change between juvenile and adult leaves (Reid & Potts 2005) and studies investigating within-plant PSM variation in some of these species also show quantitative differences in PSMs between leaf types (Gras *et al.* 2005; Loney *et al.* 2006a). While there are quantitative differences between leaf types, the genetic-based expression of PSMs across these life stages appears to be consistent (O'Reilly-Wapstra *et al.* 2007).

2.5.3 The effect of water limitation on within-plant PSM concentrations

Developing eucalypt leaves of juvenile plants that expanded during the water availability treatments maintained PSM concentrations comparable to developing leaves of control plants, with the exception of geraniol and bicyclogermacrene concentrations which decreased in specific foliage age class groups. These two

compounds appear to play a role in the interactions between plants and invertebrates. For example, while geraniol can be toxic to invertebrates (Gallardo *et al.* 2012) it also acts as an attractant to beetles (Karunaratne *et al.* 2008), and bicyclogermacrene influences aphid alarm behaviour (Bruce *et al.* 2005). However, concentrations of geraniol and bicyclogermacrene within these *E. globulus* and *E. viminalis* leaves are relatively low (Table 3) and, therefore, we are unsure of the biological impact that decreasing concentrations of these compounds during water deficit may have.

2.5.4 Ecological impacts of water limited Eucalyptus

Plant secondary metabolites in eucalypts influence many ecological interactions including browsing (Wiggins *et al.* 2006a; O'Reilly-Wapstra *et al.* 2010; Youngentob *et al.* 2011) flammability (Holmes 2009) and litter decomposition (Horner *et al.* 1988). Using a narrow range of genetic material from two *Eucalyptus* species, we found only limited impact of reduced water availability on these PSM concentrations in two eucalypt species. Our results align with a meta-analysis showing that drought generally does not impact the foliar concentrations of many PSMs including flavonoids, phenolic glycosides, CTs, mono- and sesquiterpenes in a range of species (Koricheva *et al.* 1998). As such, increasing drought periods may not have major impacts on PSM mediated ecological interactions such as koala browsing on eucalypts (Moore *et al.* 2005). We conclude that the publication of more studies incorporating a range of water treatments, diverse intra-specific plant genotypes and PSM classes would enhance our understanding of specific plant responses to drought, and enable broad response patterns across systems to be identified.

Chapter 3

Responses to mild soil water deficit and re-watering differ among secondary metabolites but are similar among provenances within *Eucalyptus* species

This chapter is accepted as:

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3.1 Abstract

Drought is predicted to become more common in many regions, and soil water deficit associated with drought can severely affect plants, and influence ecological interactions involving plant secondary metabolites (PSMs). We tested whether soil water deficit and recovery would differentially impact physiological, morphological and chemical traits of *Eucalyptus globulus* and *E. viminalis* from multiple provenances located across a rainfall gradient that also differ in drought tolerance. Both species and all provenances were similarly affected by water deficit and recovery regardless of genetic-based differences, and only foliar ABA levels differed among provenances during water deficit. Across all species and provenances, water deficit increased LMA and condensed tannins concentrations, decreased Ψ_{leaf}, above-ground biomass and concentrations of formylated phloroglucinol compounds (FPCs). Recovery reduced leaf C:N, total phenolic and chlorogenic acid concentrations. Neither water deficit nor recovery affected total oil yield or individual terpene concentrations. Levels of trait plasticity were far less than levels of constitutive trait variation among provenances. The uniform and moderate effects of water deficit on eucalypt traits suggest that a widespread and uniform drought will influence provenances equally across each species range. However, spatial and temporal drought heterogeneity coupled with variable drought severity will result in equally heterogeneous intra-specific drought responses.

3.2 Introduction

The changing global climate is predicted to alter rainfall patterns, and decreasing rainfall in many regions will lead to more frequent, prolonged and/or severe drought events (Collins *et al.* 2013). Drought causes soil water deficit, and during soil water deficit plants need to balance the uptake of CO_2 against the loss of water through stomata (Maximov 1929; Brodribb & Cochard 2009). Soil water deficit leading to long-term stomatal closure can result in a negative carbon balance as plant carbon stores are depleted (McDowell *et al.* 2008). Water deficit can also affect cellular metabolism, as increased concentrations of reactive oxygen species (ROS) damage proteins, lipids, carbohydrates and DNA (Bandurska *et al.* 2013). Drought tolerance varies among plant species, as some species have developed physiological mechanisms and morphological traits that reduce the potential for water stress or enable tolerance of water deficit (e.g. Bréda *et al.* 2006; McDowell *et al.* 2008; Blackman *et al.* 2010; Nardini & Luglio 2014). Drought tolerance also varies within-species, including among populations of a species naturally found over a wide area (e.g. Tuomela 1997; Dutkowski & Potts 2012; Taeger *et al.* 2013; Cochrane *et al.* 2014; de la Mata *et al.* 2014).

Plant species occurring over a wide environmental gradient can adapt to local environments, so that individuals of a species from drier or more drought-prone regions are less impacted by drought than individuals from wetter regions (Taeger *et al.* 2013; Anderegg 2015). For example, genotypes of *Fagus sylvatica* (European beech) from dry regions are more drought tolerant than *F. sylvatica* from wetter regions of the species distribution (Thiel *et al.* 2014). Likewise, within-species drought tolerance variation has been described in numerous species including *Eucalyptus microtheca* (Tuomela 1997; Li *et al.* 2000) and *E. globulus* (Dutkowski & Potts 2012), as well as in *Pinus* (Tognetti *et al.* 1997; Chambel *et al.* 2007) and European grasses (Beierkuhnlein *et al.* 2011). However, intra-specific variation in drought tolerance does not always correlate as expected with rainfall metrics (e.g.

Beierkuhnlein *et al.* 2011; Lamy *et al.* 2011; Cochrane *et al.* 2014), and patterns of response to drought can vary among species of the same genus (e.g. *Pinus* spp. Chambel *et al.* 2007). Drought influences plant traits which are involved in plant tolerance or avoidance of water stress (e.g. foliar abscisic acid [ABA] level; Tardieu & Simonneau 1998; Bauer *et al.* 2013; McAdam & Brodribb 2014), yet drought can also influence plant traits which have diverse effects outside of the plant.

Plant secondary metabolites (PSMs) are compounds found throughout plant tissues (Wink 2010) which can have important ecological influences such as contributing to the decomposition, flammability and defence of plants or plant parts (Wiggins *et al.* 2006a; Ormeño *et al.* 2009; Wink 2010; Youngentob *et al.* 2011; Chomel *et al.* 2014). The impact of PSMs can be concentration dependant (Jensen *et al.* 2014), and PSM concentrations can naturally vary among provenances within plant species (Moore & Foley 2005; O'Reilly-Wapstra *et al.* 2010; McKiernan *et al.* 2012). The concentrations of many PSMs are plastic, and vary due to environmental variables such as low water availability (Miles *et al.* 1982; Gleadow & Woodrow 2002; Zhang *et al.* 2012), however, PSM accumulation under water deficit can be species-, experiment-, PSM class- or even compound-specific (Pizarro & Bisigato 2010; Zhang *et al.* 2012). Furthermore, the effect of water deficit on PSM concentrations may differ among provenances of a species which vary in their tolerance of water deficit, resulting in diverse impacts on PSM mediated ecological interactions across a plant species range. To unravel the complexity of species × water deficit × PSM interactions, research that incorporates within-species provenances that vary in both drought tolerance and constitutive PSM concentrations will aid our understanding of overall species responses, provenance level responses, and flow-on ecological effects (Tognetti *et al.* 1997; Staudt *et al.* 2008; Ait Said *et al.* 2011).

Eucalyptus is a dominant tree genus consisting of over seven hundred species (Brooker 2002), and leaves contain carbon- and nitrogen-based PSMs which vary qualitatively and quantitatively among *Eucalyptus* species (Li *et al.* 1996; Nicolle *et al.* 1998; Steinbauer 2010). PSMs also vary quantitatively among genetically distinct provenances within *Eucalyptus* species, usually at the broad scale between geographically separated locations (O'Reilly-Wapstra *et al.* 2010; Andrew *et al.* 2013), but also among individual trees within a location (Moore & Foley 2005). *Eucalyptus* species often grow naturally over a vast area, and considerable environmental variation can exist across a species range (Austin *et al.* 1990; Warren *et al.* 2005; Andrew *et al.* 2007b). Previously, *E. globulus* (Dutkowski & Potts 2012) and *E. viminalis* (Ladiges 1974) genetic-based drought tolerance was shown to vary among localities. As such, variable drought tolerance combined with intra-specific variation of genetic-based PSM concentrations and natural environmental gradients make *Eucalyptus* an ideal genus to study intra-specific responses to water deficit on multiple plant traits.

Here, juvenile *E. globulus* and *E. viminalis* provenances from the same four locations across the species' natural range were used in a glasshouse-based drought experiment, incorporating a water deficit treatment and also a recovery period. The two week recovery period (water to field capacity) was included primarily to assess the effect of re-watering after water deficit on leaf chemical traits. Previous work showed that most assayed carbon-based PSMs (certain terpenes and phenolics) remained stable in juvenile plants of these species subjected to soil water deficit (Chapter 2). However, this previous work used seed from one location per species, and limited inferences could be made about species level responses, or variation in responses within species. Here we used multiple provenances per species in order to test if the findings in Chapter 2 were representative of juveniles of each species, or if genetic variation among provenances and environmental variation among locations would lead to a range of responses to water deficit. We hypothesised that when provided with a standardised but limited amount of water, eucalypts of both species

from the drier locations would tolerate water deficit and experience less water stress, and that plant traits would be less affected, compared with the responses of eucalypts from wetter locations. We also hypothesised that re-watering (recovery) would reverse the effects that water deficit had on concentrations of selected PSM and that after recovery PSM concentrations would be similar to those of controls. The specific aims of this study were to determine if: 1) constitutive plant traits of these two species differed among localities; 2) responses to water deficit differed depending on locality; 3) traits of different types (morphological, chemical, chemical class) were differentially affected by water deficit and; 4) relief from soil water deficit (two weeks re-watering) would affect chemical plant traits.

3.3 Materials and Methods

3.3.1 Plant material

Eucalyptus globulus and *E. viminalis* are phylogenetically closely related within *Eucalyptus* subgenus *Symphyomyrtus* (Steane *et al.* 2011). *Eucalyptus globulus* and *E. viminalis* provenances occur sympatrically at each of the four locations (Fig.1), and while genetic variation in mature *E. globulus* drought tolerance has been reported among these localities (Dutkowski & Potts 2012), the drought tolerance of *E. viminalis* from these sites is unknown. Open-pollinated *E. globulus* and *E. viminalis* seed were collected from seven trees per species at each location (Southern Tasmania [ST], Queens Domain [QD], St Helens [SH] and King Island [KI]), totalling 28 seed trees per species (Table 1). Seed obtained from a single tree was pooled, and the adult tree of origin was termed the 'mother' while seed from a single mother tree was termed a 'family'. Mother trees of each species were chosen from across each location, and were at least 100 m apart to avoid sampling highly related individuals and to include the genetic variation among trees within each provenance. Mother trees on KI were spread across the island.

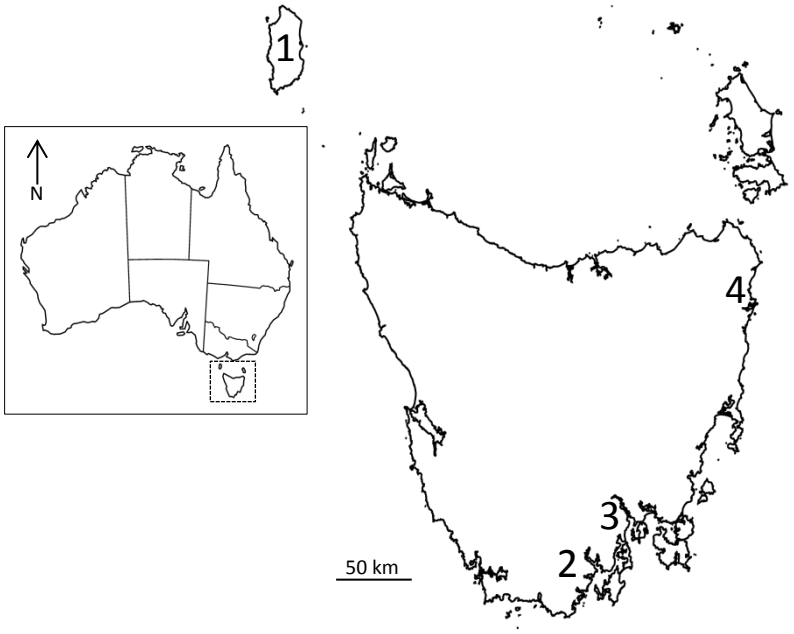

Figure 1. Location of four sites where open-pollinated *Eucalyptus globulus* and *Eucalyptus viminalis* seed were collected. Inset shows location of Tasmania (within dashed box) relative to mainland Australia. Expanded view of Tasmania shows position of King Island (1), Southern Tasmania (2), Queens Domain (3) and St Helens (4) localities.

Table 1. Details of four localities from which *Eucalyptus globulus* and *E. viminalis* open-pollinated seed were collected for use in a water deficit experiment[1].

	Locality			
	KI	**ST**	**QD**	**SH**
Co-ordinates - latitude	-39.865881°	-43.028629°	-42.864423°	-41.271634°
-longitude	143.980070°	146.890798°	147.322510°	148.312977°
Mean altitude (m) - *E. globulus*	35	326	102	117
- *E. viminalis*	27	263	120	28
Mean annual rainfall (mm)$^{+}$	927.4	960.0	601.6	817.72
Mean number of days of rain$^{\#}$	162.6	176.2	58.7	152.1
Rainfall driest quarter (mm)$^{+}$	126.7	179.2	126.6	180.5
Rainfall wettest quarter (mm)$^{+}$	350.0	309.5	174.8	234.2
Rainfall seasonality (C of V)$^{+}$	37.8	20.9	15.0	14.7
Drought susceptibility^	Highly susceptible	Susceptible	Intermediate	Tolerant
Mean maximum temperature (°C) $^{\#}$	16.8	16.9	17.0	17.9
Mother trees per species	7	7	7	7
Individuals per mother	8	8	8	8
Total no. plants grown	112	112	112	112

[1] $^{\#}$ data obtained from closest Bureau of Meteorology (Australia) weather station
$^{\wedge}$ from Dutkowski and Potts (2012)
$^{+}$ data from ANUCLIM (Xu & Hutchinson 2010) using geographic coordinates.
KI King Island, *ST* Southern Tasmania, *QD* Queens Domain, *SH* St Helens

Eucalyptus globulus and *E. viminalis* seed from all 56 mother trees (28 mothers per species) were germinated and grown for 1 month in a naturally lit glasshouse. Uniform size (cotyledons and one leaf pair) seedlings were selected (eight individuals per family) and transplanted into individual plastic pots (base 38 x 38 mm, top 50 x 50 mm, height 118 mm). Potting mix contained eight parts composted fine pine bark: three parts coarse river sand, and N:P:K [19: 2.6: 10] at 1 g^{-1} /L potting mix. The pH was adjusted to approximately 6.0 with the addition of dolomite lime at 3 kg/m^{-3}. Seedlings were grown for 10 weeks, then re-potted into larger pots (base 115 mm diameter, top 138 mm diameter, height 250 mm) containing exactly 900 g of moist potting mix (430 g^{-1} DW [dry weight]) with the addition of extra fertiliser (5 g Osmocote® 3-4 month [N14:P6.1 :K11.6] per pot) and wetting agent (Everris Hydraflo® at 1.35 L/m^{-3} potting mix). All 448 pots (56 families × eight seedlings) contained equal bulk density of potting mix and a single plant. Potted plants were set out in a randomised split-plot design, with 12 plots in total. Each plot contained a control block and a water deficit treatment block, with treatments separated into two blocks to simplify watering. Each plot (n=12 plots total) contained equal numbers of *E. globulus* and *E. viminalis* from each locality (randomly selected with regard to family), yet some plots contained 32 plants (2 plants per treatment [n=2] per locality [n=4] per species [n=2]) while others contained 48 plants (3 plants per treatment per locality per species). Plants were randomised within blocks, a control block and a water deficit block were located next to each other to create one plot, and the 12 plots were randomised within the glasshouse. Plants of all species and localities in all plots were grown for a further 7 weeks and watered daily to field capacity before experimental treatments were applied.

3.3.2 Experimental treatments

Treatments began in January 2013 (summer) when eucalypts were 26 weeks old and at least 50 cm tall. Water loss through evapotranspiration was calculated

separately for each species (Mitchell *et al.* 2013). Daily evapotranspiration was monitored gravimetrically from a subset of control plants (n=6 per species). A percentage (50%) of the three day rolling mean evapotranspiration of controls (of each species) was calculated every second day, then provided to water deficit plants of that species. The control plants used to determine evapotranspirational water loss were selected randomly, and were randomly re-selected weekly. Control plants of both species continued to be watered to field capacity daily. Following this method, all plants of a species in the water deficit treatment were provided the same amount of water, regardless of the water usage of each individual water limited plant (Mitchell *et al.* 2013). Therefore, rather than creating a uniform level of water stress, we provided a uniform level of water availability to detect genetic-based variation in resource utilisation and allocation. Due to the hydrophobic nature of dry potting mix, plastic bags were placed over the base of all water deficit plants to eliminate drainage when watered, while the pot remained open to facilitate evaporation. Plastic bags did not cause water-logging.

Treatments were maintained for 3 months, and then six of the 12 randomised plots (each containing a control and water deficit block) were harvested (Harvest 1) to provide samples from control and water deficit plants for investigation of water deficit impacts on plant traits (Fig. 2). Plastic bags were then removed from all remaining plants of the water deficit treatment, and these plants were watered daily to field capacity for two weeks along with remaining controls in each plot to enable plant recovery (Fig. 2). After two weeks, fully watered eucalypts in the remaining six split-plots (six control blocks + six water limited/recovered blocks) were harvested (Harvest 2). The samples collected at Harvest 2 were used to investigate trait variation between control plants and those eucalypts which had recovered from water deficit (Fig. 2). *Eucalyptus* developmental changes could be assessed between the controls sampled at Harvest 1 and Harvest 2 (two weeks difference), and if no developmental changes were found, the impact of recovery could be assessed using water limited plants (Harvest 1) and recovered plants (Harvest 2; Fig. 2). Finally, the

eight individual plants from each family were evenly but randomly divided among two treatments (control and water limited), and the two sampling periods (Harvests 1 and 2; Fig. 2).

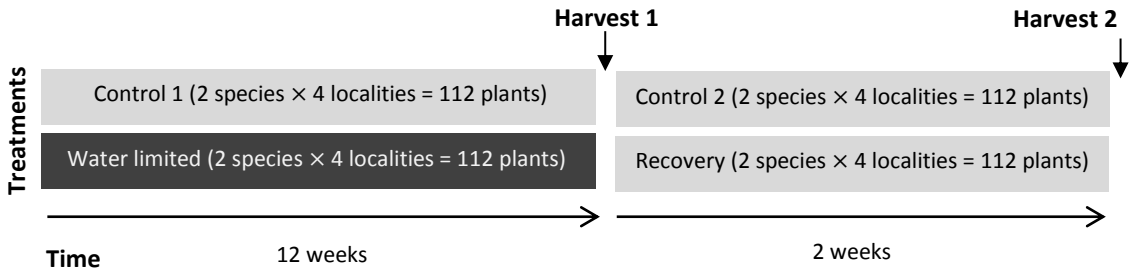

Figure 2. Experimental plan. A total of 448 eucalypts were used in the experiment, with 224 plants sampled at Harvest 1 (control and water limited treatments), then the remaining 224 plants sampled at Harvest 2 (control and recovered treatments).

3.3.3 Sample collection

Due to the number of plants being sampled at Harvest 1 (n=224) and then at Harvest 2 (n=224), the plants at each Harvest were allocated to one of 14 sampling replicates, each containing 16 plants (one individual [of a random mother] per locality × four localities × two species × two treatments = one sampling replicate). The 14 sampling replicates in each harvest were sampled over six days. Plants were destructively sampled at Harvest 1 (control and water deficit), and then two weeks after Harvest 1 was completed, the remaining 14 sampling replicates were destructively sampled at Harvest 2 (control and recovered). The only differences between sampling at Harvests 1 and 2 were that; (1) water limited plants were not watered the morning of sampling during Harvest 1 (controls were fully watered), while at Harvest 2 all plants were fully watered that morning and; (2) samples for foliar abscisic acid (ABA) analysis (all plants) and leaf water potential (Ψ_{leaf}; 143 plants) were collected at Harvest 1 (controls and water limited plants), while limited samples were taken at Harvest 2 when all plants were fully watered. Foliar ABA levels (a phytohormone which signals stomata closure during water limitation) and vascular tension of all plants was expected to be similar to control levels at Harvest 2 regardless of previous treatment (Correia *et al.* 2014). Sampling on each day of each harvest followed the protocol described below.

Plants were placed outside in full sunlight (1500 – 2000 µmol m^{-2} s^{-1}) for >30 min prior to sampling (between 11.30am and 2.00pm). A subset of plants of each species, locality and treatment had been selected *a priori* for measurement of Ψ_{leaf} (Harvest 1 = 143 individual plants, Harvest 2 = 19 plants), which was measured on a single fully-expanded leaf from the same position on each plant using a Scholander pressure chamber (PMS, Albany, OR, USA). Next, fully-expanded juvenile leaves were taken from multiple positions (all fully-expanded leaves of a plant were of the same ontogenetic stage) on each plant (~eight leaves per *E. viminalis*; three leaves per *E. globulus* due to leaf size differences) to quantify foliar ABA levels, which we

used as a proxy for stomatal adjustment during water stress (McAdam & Brodribb 2015a). Leaves for ABA analysis taken from an individual plant were considered to represent the sampled plant. Fully-expanded leaves (~3 g total) representative of three plants (same species, locality and treatment) were pooled and immediately immersed in liquid nitrogen, then stored at -70°C for ABA quantification.

Remaining parts of each plant were then destructively sampled in turn. First, a single fully-expanded leaf was removed from the 4th node below the apex (position chosen for consistency) and was frozen (-12°C) for morphological assessment. Next, the stem was cut at soil level and total fresh above-ground biomass was determined. From this cut stem, only the top (youngest) eight fully-expanded *E. globulus* leaf pairs or the top 50% of fully-expanded *E. viminalis* leaves were harvested, as we were only interested in leaves which developed during the treatment period. These leaves were stripped from the stem, pooled and mixed, disregarding leaves which were not fully-expanded or showed signs of damage. A random sub-sample of these leaves were frozen for essential oil analysis, then the remaining pooled leaves were weighed, frozen, freeze dried, re-weighed, scanned using near infrared spectroscopy (NIRS; see below), ground in a Cyclotec™ 1093 cyclone mill (Foss, Hillerød, DK) and passed through a 1 mm sieve for analysis of primary chemistry (C and N levels; Thermo Finnigan EA 1112 Series Flash Elemental Analyser, Italy), total phenolics (TPs), condensed tannins (CTs), and formylated phloroglucinol compounds (FPCs). Sample mass prior to and post lyophilisation was used for quantification of fresh leaf water content which was expressed as percentage fresh weight (% FW). The remaining above-ground plant section (fresh stem and un-harvested leaves) was weighed and oven dried at 40°C until constant mass. Total dry above-ground biomass was determined using the resulting dry stem and leaf mass, plus the calculated dry mass of harvested leaves using the corresponding leaf water content of each sample. The below-ground portion of each plant remained in the pot, including the lignotubers. Lignotubers are storage organs developed from cotyledonary and early seedling leaf nodes from which regenerative shoots develop after disturbance

(e.g. fire or drought) (Whittock *et al.* 2003). The lignotuber was located, the stem was cut 1 cm below the lignotuber, and the sample frozen for morphological assessment. Roots were discarded.

3.3.4 Foliar abscisic acid (ABA)

Eighty pooled ABA samples from Harvest 1 were assayed (five pooled samples × two species × four localities × two treatments [control and water limited]). Extraction of foliar ABA followed the method described by McAdam and Brodribb (2014) using an internal standard, except that ~0.2 g of pooled leaves were used per sample. Endogenous ABA was physicochemically quantified using a Waters ultra-performance liquid chromatograph coupled to a Xevo triple quadrupole mass spectrometer (UPLC-MS), with results expressed as ng g^{-1} FW.

3.3.5 Lignotuber and leaf dimensions

Lignotuber size was calculated for all plants (n=448) using the maximum lignotuber width minus stem diameter, and expressed as the ratio of lignotuber to stem (Ladiges & Ashton 1974). Leaf mass per unit area (LMA) was calculated on a single leaf from the 4^{th} node below apex on all plants using the dry mass and lamina area of each leaf. Leaf area was determined using a scanned leaf image and ImageJ (version 1.48). LMA was expressed as g m^{-2}.

3.3.6 Secondary chemistry

Eucalypt foliage contains a wide range of PSMs, and we assayed selected non-nitrogen containing chemical groups and individual compounds which have known ecological roles (Wiggins *et al.* 2006a; Ormeño *et al.* 2009; Youngentob *et al.* 2011; Chomel *et al.* 2014) to investigate the impact of soil water deficit and recovery on concentrations of these compounds. Due to the large number of samples (c. 800)

from this and two other concurrent experiments (same mother trees, plant age, glasshouse; Chapters 4-5), a subset of samples from the three experiments were used for chemical assays, then the chemical data was correlated to NIR spectra to create independently validated calibration models for each trait (see below). The subset of samples used in chemical assays included equal quantity of samples from each species, locality and treatments, and these were selected randomly. The model for each chemical trait was used to predict the remaining dataset for all samples in the three experiments (Chapters 3-5) based on NIR spectra of those samples

Extraction, analysis, identification and quantification of the most abundant essential oil components (1,8-cineole, α-pinene, globulol, aromadendrene and limonene) followed the methods outlined by McKiernan *et al.* (2012). Concentrations of 1,8-cineole and α-pinene were expressed as mg g^{-1} DW using standards, while total oil yield and other oil components were expressed as mg g^{-1} DW cineole equivalents using the dry weight of each individual sample. Phenols were extracted, and then TPs and CTs were quantified in duplicate as described in Chapter 2, with the exception that a VIS-7200A UV-visible spectrophotometer (Techcomp Limited, Hong Kong) was used. Total phenolics were expressed as mg g^{-1} DW gallic acid equivalents, CT results were expressed as mg g^{-1} DW sorghum tannin equivalents using purified sorghum tannin (Hagerman & Butler 1980). We assayed three specific FPCs (macrocarpals A and G and sideroxylonal A) by High Performance Liquid Chromatography (HPLC) following methods of Wallis and Foley (2005). A group of relatively late-eluting FPCs (eluting just before and after macrocarpal G) were also quantified. Macrocarpal A and sideroxylonal A were expressed as mg g^{-1} DW, while macrocarpal G and the group of late-eluting FPCs was expressed as mg g^{-1} DW macrocarpal A equivalents, using standards described by Eyles *et al.* (2003a).

3.3.7 Chlorogenic acid

In a previous experiment (Chapter 2), water limitation reduced TP concentrations of juvenile *E. viminalis*, while CT concentrations remained stable. Here, we investigated this further by identifying specific compounds in the aqueous acetone extracts from leaves (Hagerman 1988). Test samples were analysed using an ultra-performance liquid chromatograph (Waters Acquity H-series UPLC) coupled to a diode array UV-Vis detector and Waters Xevo triple quadrupole mass spectrometer. The mobile phase was a gradient from 1% acetic acid to 45:55 acetonitrile/1% acetic acid at 15 minutes. Chromatograms at 370 nm and 280 nm were extracted from the diode array data. We identified major UV peaks at six retention times which appeared to correlate with the treatments, and MS data showed that each peak consisted of one main compound. Of these, chlorogenic acid and pedunculagin were positively identified using a combination of UV-vis spectra, MS data and direct comparison with authentic standards. Two compounds were tentatively identified as eucaglobulin (*m/z* 497) and quercitin glucuronide (*m/z* 477). The remaining two compounds were a galloylglucose (*m/z* 331) and either luteolin rutinoside or kaempferol rutinoside (*m/z* 593). Only chlorogenic acid was further investigated as it was one of only two positively identified compounds, and chlorogenic acid has known roles in alleviating oxidative water stress damage (Živković *et al.* 2010) and is therefore, of particular interest here.

To quantify chlorogenic acid concentrations, phenols were extracted from the 204 dried and ground samples using aqueous acetone as described above to enable comparison with TP and CT data, as well as with previous work (Chapter 2). Supernatant of each sample was passed through a new 0.22 µm nylon syringe filter directly into a 2 mL auto-sampler vial for analysis using UPLC/UV-Vis. Chlorogenic acid was quantified using a Waters Acquity H-series UPLC, Waters Acquity Photo Diode Array (PDA) detector, an Acquity BEH C18 1.7µM column (2.1 × 100 mm), a chromatogram at 325 nm (retention time 1.18 min) and a pharmaceutical grade

chlorogenic acid reference standard (Sigma-Aldrich). A flow rate of 0.35 mL/min and a mobile phase gradient of 90%A/10%B to 50%A/50%B (mobile phase A [1% acetic acid] and mobile phase B [acetonitrile]) at 3 min followed by 3 min requilibration was used. Results were expressed as mg g^{-1}DW.

3.3.8 Near infrared spectroscopy (NIRS)

All freeze-dried whole leaf samples (c. 800 samples from concurrent experiments described in Chapters 3-5) were measured using the standard optical fibre of a Bruker MPA Fourier Transform NIR spectrometer (Bruker, Germany) and a TE-InGaAs NIR detector. Two different positions (at either end of leaf on opposite sides of midrib) were measured on two different leaves per sample and averaged to one single spectrum. Each spectrum was recorded using a spectral range between 12500 – 4000 cm^{-1} with a spectral resolution of 8 cm^{-1} and 4 scans. Background spectra were measured using 64 scans on the standard Bruker reference for the fibre optic cable which were used for a subsequent maximum of 2 hours. Quantitative chemical data combined with the corresponding NIR spectra for each sample were used to create a partial least squares (PLS) calibration model for each trait using The Unscambler X (version 10.1, CAMO software, Norway). The models for total oil, 1,8-cineole, α-pinene, globulol, aromadendrene and limonene used 156 samples, and were randomly cross-validated using 20 segments (different random subsamples of the 156 total samples). Modelling all other traits used 204 samples total, with 154 samples randomly selected for model calibration and 50 samples used for validation. Spectra and chemical data for each trait were checked for clustering at the experiment, species, locality, treatment or harvest level using principal component analysis and no clustering was identified. Spectral pre-treatment were used to optimise the individual PLS calibration models with most using a combination of multiplicative scatter correction or normalisation with Savitzky-Golay smoothing or first derivatisation (Table 2). The sideroxylonal A model performed poorly during validation and this compound was excluded from further analysis (Chapters 3-5). Models for all other chemical traits performed well during validation (Table 2), for

each trait model the root mean squared error of prediction (RMSEP) was only marginally higher than mean lab errors calculated from triplicate samples. NIRS spectra of remaining samples were used to predict chemical values for each compound in turn. The complete dataset (assayed chemical data and NIRS predicted values) were then separated by experiment into three datasets. Data for this experiment was analysed as follows.

Table 2. Details of predictive models calibrated using near infrared spectra (NIRS) and complimentary laboratory assayed chemical data for each chemical trait in *Eucalyptus* leaves[2]

Model	No. calibration samples	No. validation samples	Factors	Spectral pre-processing	Smoothing points	RMSEV	r^2	Bias
1,8-Cineole	156	C-Val	8	MSC+1stD	25	4.47	0.87	-0.01
α-Pinene	156	C-Val	18	MSC+1stD	25	1.74	0.85	0.03
Aromadendrene	156	C-Val	7	MSC+1stD	17	0.92	0.78	-0.02
Globulol	156	C-Val	7	MSC+1stD	9	0.50	0.78	<0.01
Limonene	156	C-Val	6	MSC+1stD	9	0.53	0.79	<0.01
Total oil	156	C-Val	8	MSC+1stD	25	8.14	0.83	-0.05
Chlorogenic Acid	154	50	9	MSC+1stD	9	1.43	0.62	-0.33
Condensed tannins	154	50	18	Smooth	17	3.52	0.64	0.14
Total phenolics	154	50	4	MSC	0	21.33	0.80	-0.90
Macrocarpal A	154	50	9	1stD	17	0.39	0.63	0.04
Macrocarpal G	154	50	10	1stD	17	1.23	0.64	0.23
Sideroxylonal A	154	50	18	Norm	0	1.80	0.54	-0.32
Non-polar FPCs	154	50	10	SNV	0	5.33	0.50	-0.24
Carbon	120	60	7	MSC+1stD	9	0.40	0.94	-0.01
Nitrogen	120	60	4	1stD	17	0.20	0.87	0.09

[2] *C-Val* Cross-Validated (random with 20 segments), *1stD* 1st derivative using Savitzky-Golay filter, *Smooth* Smoothing using Savitzky-Golay filter, *SNV* Standard Normal Variate, *MSC* Multiplicative Scatter correction, *Norm* Normalisation, *RMSEV* Root Mean Standard Error of Validation. *Bias* (indicating systematic errors) of measured and predicted values. Samples for model calibration were randomly selected, but were chosen to include equal numbers of samples from each species (*E. globulus* and *E. viminalis*), locality and treatment. The resulting global predictive model for each chemical trait was then used to predict values on samples regardless of species, provenance or treatment (except 0% water samples; Chapter 5).

3.3.9 Statistical analysis

All analyses were completed using SAS statistical software package (version 9.2, SAS Institute Inc., Cary USA). Testing of fixed effects (species, locality, treatment and sampling time [harvest]) was undertaken using various general linear models (PROC GLM of SAS) depending upon the data set. A large number of Ψ_{leaf} data was collected at Harvest 1, and so species (*E. globulus* and *E. viminalis*), locality (KI, ST, QD and SH), treatment (control and water limited) and their interactions were fitted in the model for Harvest 1. Ψ_{leaf} data from Harvest 2 were analysed separately due to the small number of samples (n=19), with only treatment fitted in the model. ABA data was collected only at Harvest 1, and samples were pooled rather than from a single plant, so only species, locality, treatment and their interactions were fitted in the ABA model. For all other traits, species, locality, treatment, Harvest (1 and 2) fixed effects and their interactions were tested in a mixed model (PROC GLIMMIX of SAS with a normal residual distribution), where mother within locality for each species (mother[locality × species]) and interactions between mother(locality × species) and harvest, or treatment, or both (harvest × treatment × mother[locality × species]) were fitted as random effects. Variation between families within each locality was also analysed separately for each species and tested using a restricted likelihood ratio test (LRT) procedure within each mixed model. Residuals for all variables were checked for assumptions of normality and heterogeneity of variances, and only ABA data were transformed to their natural logarithm. Due to the number of individual mixed model analyses performed (n = 17), the false discovery rate (FDR) was controlled following Benjamini and Hochberg (2000), and FDR control was again used for LRT (mother[locality × species]) and associated interactions.

3.4 Results

3.4.1 Water potential (Ψ_{leaf})

At Harvest 1 (control and water limited treatments), the mean Ψ_{leaf} of water limited plants (-0.88 MPa) was lower than the mean Ψ_{leaf} of control plants (-0.24 MPa; $F_{1,65}$=56.1; P<0.001), indicating that the treatment was effective but not highly stressful (midday Ψ_{leaf} of severely water stressed *E. globulus* can reach c. -2.3 MPa; Mitchell *et al*. 2014). No difference in mean Ψ_{leaf} was detected between species ($F_{1,65}$=0.02; P=0.90) or among localities ($F_{3,65}$=1.75; P=0.15), and no significant interactions were detected, indicating that both species at each locality responded similarly to the standardised level of sub-optimal water. When the second group of eucalypts were sampled two weeks later at Harvest 2, no significant difference was detected between the mean Ψ_{leaf} of control and recovered plants ($F_{1,10}$=0.21; P=0.66). The mean Ψ_{leaf} at Harvest 2 was -0.35 MPa, calculated from both control and recovered plants.

3.4.2 Foliar abscisic acid (ABA)

Foliar ABA level was compared between treatments, species, localities and all interactions after three months of continuous treatment (Harvest 1). Foliar ABA level was used as a proxy for stomatal adjustment during water stress. Overall, the water deficit treatment increased foliar ABA levels compared to ABA levels in control plants (692.5 and 459.1 ng g^{-1} FW respectively; $F_{1,74}$=34.17; P<0.001), and the foliar ABA levels in both species were altered similarly by water deficit (no species × treatment interaction; $F_{1,74}$=0.45; P=0.50). Specifically, water deficit increased *E. globulus* foliar ABA levels by 67% (376.4 in controls to 626.8 ng g^{-1} FW in water stressed plants), and increased *E. viminalis* foliar ABA levels by 40% (541.8 in controls to 758.1 ng g^{-1} FW in water stressed plants). However, variation in foliar ABA levels were primarily explained by differences between species ($F_{1,74}$=13.53; P<0.001) and also by a significant 2-way interaction involving

treatment and locality main effects ($F_{3,74}$=2.87; P=0.04). First, *E. viminalis* leaves contained higher levels of foliar ABA than *E. globulus* leaves (649.9 and 501.6 ng g^{-1} FW respectively). Second, the responses to water deficit varied among localities (Fig. 3). Large increases to foliar ABA levels were observed in leaves of juvenile eucalypts from ST (wet locality; 908 ng g^{-1} FW) and SH (dry locality; 631 ng g^{-1} FW) due to soil water deficit compared to controls (457 and 383 ng g^{-1} FW, respectively), Foliar ABA levels in leaves of juvenile eucalypts from QD (dry locality; 594 ng g^{-1} FW) and KI (wet locality; 636 ng g^{-1} FW) did not significantly change during soil water deficit from control levels (410 and 586 ng g^{-1} FW, respectively). While the effect of water deficit on ABA levels in leaves from KI eucalypts was minimal (P=0.99), the effect of water deficit on ABA levels in QD leaves was larger, yet still non-significant (P=0.20). No significant species × locality × treatment interaction influenced foliar ABA levels ($F_{3,74}$=0.31; P=0.82), however, this interaction is presented as Figure 3 to enable species responses to be viewed within the significant locality × treatment interaction.

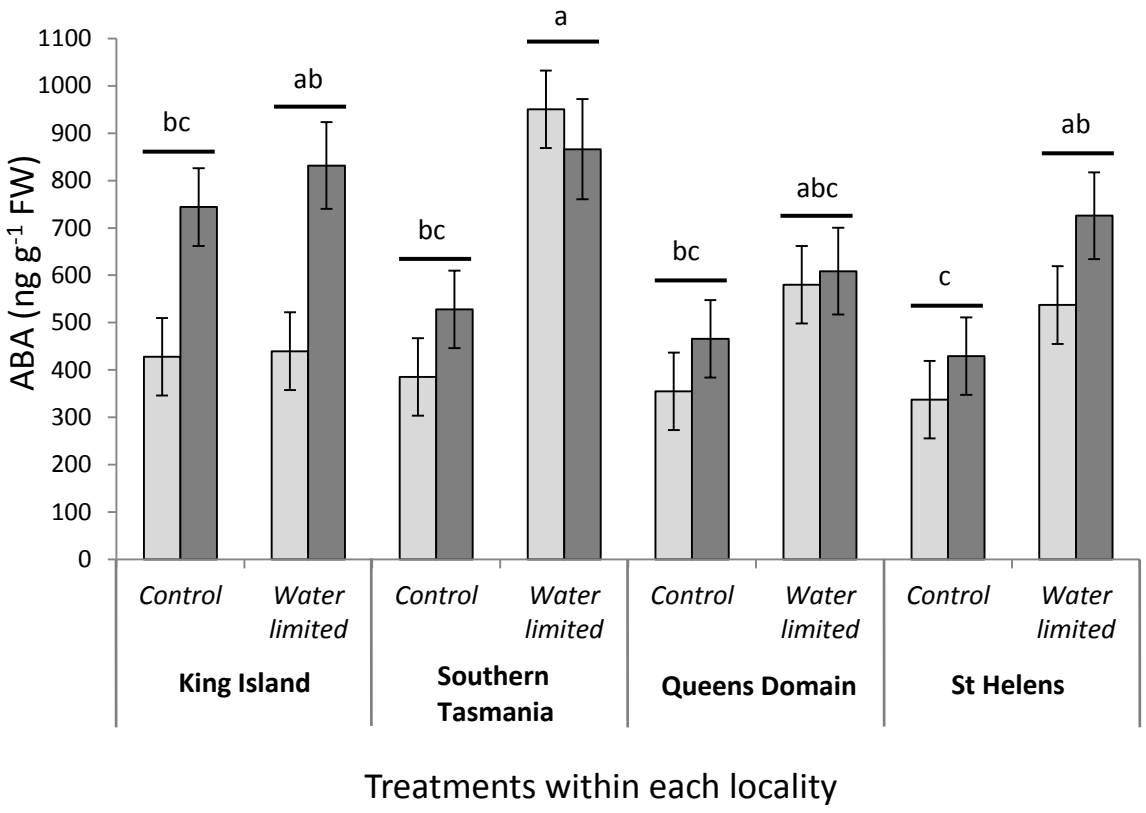

Figure 3. Least squares mean (LSmean) foliar abscisic acid (ABA) level in leaves of juvenile *Eucalyptus globulus* (light bars) and *E. viminalis* (dark bars) from four localities grown under control or water limited (provided 50% of the control plant evapotranspirational water loss) treatments. Error bars are standard error (±SE). Letters indicate significant differences ($P \leq 0.05$) among all treatment and locality combinations (disregarding species within each treatment) after Tukey's *post hoc* tests. No significant species × locality × treatment effect was detected ($F_{3,74}=0.31$; $P=0.82$), yet the interaction is shown to illustrate species foliar ABA levels in relation to the significant locality × treatment interaction ($F_{3,74}=2.87$; $P=0.04$).

3.4.3 Results of the main mixed model analysis

The remainder of plant traits (n = 17) were analysed in turn using a mixed model analysis with main effects and interactions involving species, locality, treatment and harvest fixed terms, and mother(locality × species) and all interactions (with treatment and harvest) as random terms. Within this full analysis, no significant 3- or 4-way interactions of the main fixed effects were detected for any trait, and so will not be presented (in Table 3) or discussed further (but see Supplemental Tables S3 and S4 for LSmeans [±SE] taken from the non-significant 4-way interactions of each trait). As such, only results for the individual main effects and 2-way interactions are presented (Table 3). To begin with, genetic-based trait variation (species, locality and species × locality interaction) will be presented. This is justified as the interactions of these genetic terms with treatment and harvest were rarely significant (Table 3). Then, the impact of treatment (water deficit) and the interaction between treatment and harvest will be presented. However, the main effect of harvest and the interaction between species and harvest will not be discussed (although results are included in Table 3) as harvest confounds the effect of time and a changed watering regime. Rather it is the treatment × harvest interaction which is of interest as this indicates a differential response of the water deficit treatment compared with the controls, and signals a recovery response (Table 3). Finally, where the treatment × harvest effect was insignificant, the impact of treatment will then be presented, with treatment defined by the mixed model analysis as 12 weeks of water deficit followed by two weeks of water to field capacity (Fig. 2), which are compared to the controls (Harvest 1 and Harvest 2 control data combined).

Table 3. Results of mixed model analyses for variation of chemical and morphological traits in juvenile *Eucalyptus globulus* and *E. viminalis* leaves between species, localities (King Island, Southern Tasmania, Queens Domain, St Helens), water treatments (control, water deficit), sampling period (Harvest 1 and 2), and 2-way interactions[3]

	Species		**Locality**		**Species × Locality**		**Treatment**		**Treatment × Harvest**		**Species × Treatment**		**Locality × Harvest**		**Locality × treatment**		**Harvest**		**Species × Harvest**	
Trait	$F_{1,47}$	***P***	$F_{3,47}$	***P***	$F_{3,47}$	***P***	$F_{1,47}$	***P***	$F_{1,47}$	***P***	$F_{1,47}$	***P***	$F_{3,47}$	***P***	$F_{3,47}$	***P***	$F_{1,47}$	***P***	$F_{1,47}$	***P***
LMA	18.3	***	1.4		0.7		0.9		17.7	***	0.1		1.6		1.2		101.6	***	12.5	***
Above-ground biomass	143.9	***	1.6		2.1		32.9	***	5.0	#	3.7		1.1		1.4		220.1	***	17.3	***
Lignotuber:Stem	10.1	**	15.2	***	3.7	*	0.8		0.5		3.2		0.2		0.6		8.3	**	0.1	
Leaf water content	138.9	***	2.1		0.9		42.0	***	109.2	***	0.4		0.7		0.1		149.2	***	7.0	*
C:N	57.32	***	3.2	#	8.48	***	28.73	***	10.38	**	1.78		0.8		2.1		38.59	***	0.16	
1,8-Cineole	8.7	**	5.0	**	1.5		2.5		1.0		0.8		0.4		0.3		54.4	***	0.6	
α-Pinene	1.7		5.5	**	2.6		1.6		0.1		0.3		0.2		0.2		7.5	*	0.0	
Aromadendrene	82.3	***	1.1		2.3		3.1		0.0		2.6		0.7		0.4		0.6		6.5	*
Globulol	100.1	***	1.5		3.1	#	2.6		0.3		2.0		0.4		0.8		1.5		3.9	#
Limonene	15.8	***	9.2	***	1.4		1.0		3.2		1.5		0.4		0.1		65.4	***	6.0	#
Total oil yield	89.3	***	7.8	***	1.0		0.1		0.1		1.0		2.2		0.1		7.0	*	0.8	
Chlorogenic acid^	73.25	***	15.88	***	2.55		1.26		15.69	***	2.87		1.3		1.3		11.00	**	2.44	
Condensed Tannins^	146.53	***	3.16	#	8.25	***	5.98	*	9.54	**	1.23		2.2		0.1		47.65	***	1.75	
Total phenolics^	115.08	***	6.75	***	9.69	***	2.09		16.12	***	0.04		2.3		1.4		67.47	***	4.68	#
Late-eluting FPCs^+	0.4		5.4	**	2.9	#	8.9	**	2.0		0.2		0.7		0.4		3.9		2.7	
Macrocarpal A^+	0.7		2.5		5.0	**	13.3	***	1.0		0.1		1.1		0.7		3.4		0.0	
Macrocarpal G^+	1.9		2.6		4.4	**	13.1	***	3.9	#	0.2		1.2		0.8		0.1		2.1	

[3] Blank *P*-values indicate no significance ($P>0.05$). *indicates $P\leq0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$.

[#] indicates significance ($P\leq0.05$) after *post hoc* tests (Tukey's) but no longer significant following control for false discovery rate (Benjamini & Hochberg 2000). *LMA* leaf mass per unit area. *Lignotuber:stem* lignotuber diameter in relation to stem diameter expressed as a ratio, *C:N* the ratio of carbon to nitrogen. Italics indicate terpene, ^ indicates phenol, +indicates formylated phloroglucinol compound (FPC). No significant ($P\leq0.05$) 3-way or 4-way interactions of the main effects were detected in the full mixed model analysis, and so only individual main effects and 2-way interactions of the main effects are presented here for conciseness. Harvest and the interaction between species and harvest (grey fill) are not discussed as without the interaction with treatment, harvest in isolation confounds the effect of time (plant development) and changes to watering regime (recovery).

3.4.4 Genetic-based trait variation

Quantitative genetic differences were detected for all traits at either species or locality level, or both (Table 3). The main source of variation in most traits was associated with differences between species, including variation in both morphological (biomass, LMA) and chemical (chlorogenic acid and terpenes) traits (Table 3). In terms of morphological variation between species (Table 3), LMA was greater in *E. viminalis* (64.5 g m^{-2}) than *E. globulus* (59.2 g m^{-2}) and above-ground biomass was greater in *E. globulus* (33.9 g^{-1} DW) than *E. viminalis* (24.5 g^{-1} DW; Table 3). Chemical traits also varied between species, as *E. globulus* leaves contained higher 1,8-cineole (23.43 mg g^{-1} DW) and chlorogenic acid (3.5 mg g^{-1} DW) concentrations than *E. viminalis* leaves (22.24 and 2.3 mg g^{-1} DW respectively). In contrast, *E. viminalis* leaves contained higher total oil yield (56.0 mg g^{-1} DW cineole equivalents), limonene (2.11 mg g^{-1} DW cineole equivalents) and globulol concentrations (2.13 mg g^{-1} DW cineole equivalents) than *E. globulus* (45.6, 1.82 and 1.63 mg g^{-1} DW respectively).

A number of chemical traits differed among localities (Table 3), and the trends of quantitative variation were parallel in both species (i.e. no species × locality interaction). Parallel chemical variation among localities was mainly found in terpene and chlorogenic acid concentrations, but also in concentrations of the late-eluting FPCs (Table 3). Concentrations of 1,8-cineole (Fig. 4a), α-pinene (Fig. 4b), limonene (Fig. 4c), total oil (Fig. 4d) and the late-eluting FPCs (Fig. 4e) were highest in eucalypts from the wetter locations (KI and ST) compared to the drier locations (QD and SH). In contrast, chlorogenic acid content was lowest in leaves from the wet KI location compared to ST (also wet), and also QD and SH (drier locations; Fig. 4f).

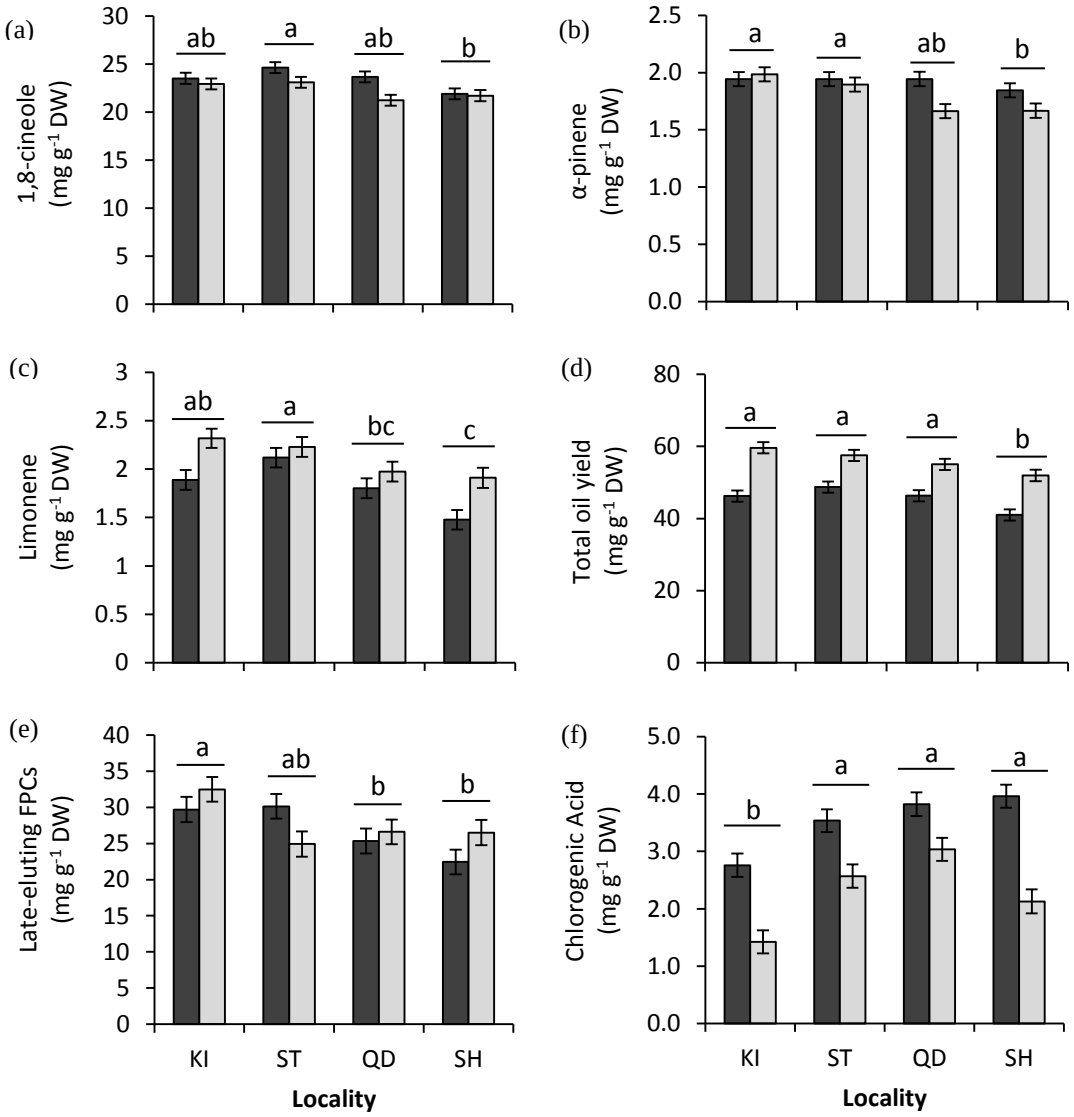

Figure 4. LSmean concentrations of (a) 1,8-cineole, (b) α-pinene, (c) limonene, (d) total oil, (e) late-eluting FPCs and (f) chlorogenic acid in juvenile *E. globulus* (dark) and *E. viminalis* (light) from four localities. *KI* King Island, *ST* Southern Tasmania, *QD* Queens Domain, *SH* St Helens. Error bars are standard error, and letters indicate significant difference ($P \leq 0.05$) after Tukey's *post hoc* test among localities. Concentrations of 1,8-cineole, α-pinene and chlorogenic acid expressed as mg g^{-1} DW. Concentrations of total oil and limonene expressed as mg g^{-1} DW cineole equivalents. Late-eluting FPCs expressed as mg g^{-1} DW macrocarpal A equivalents. Note different scales on y-axis among individual graphs.

Patterns of variation in the concentration of major phenol groups and the macrocarpals differed among localities, and the pattern was different for the two species (species × locality interaction; Table 3). Firstly, while CT (Fig. 5a) and TP concentrations (Fig. 5b) did not vary among *E. globulus* from the four localities, CT and TP concentrations in *E. viminalis* leaves did vary among localities. Condensed tannin and TP patterns of variation in *E. viminalis* did not comply with a wet and dry location dichotomy as seen in terpene concentrations, instead CT concentrations were highest in leaves of KI and QD *E. viminalis* (Fig. 5a), while TP concentrations were greatest in SH, intermediate in ST, and lowest in KI and QD *E. viminalis* (Fig. 5b). Macrocarpal (A and G) concentrations did not vary overall between species, or among localities, but did vary between eucalypt species from ST, where *E. globulus* had higher concentrations of both macrocarpals compared to *E. viminalis* (Fig. 5c, d). There was also variation in *E. viminalis* macrocarpal concentrations among localities, as both macrocarpals were higher in *E. viminalis* from KI than in those from ST (Fig. 5c, d). Macrocarpal concentrations in *E. globulus* leaves did not differ among localities.

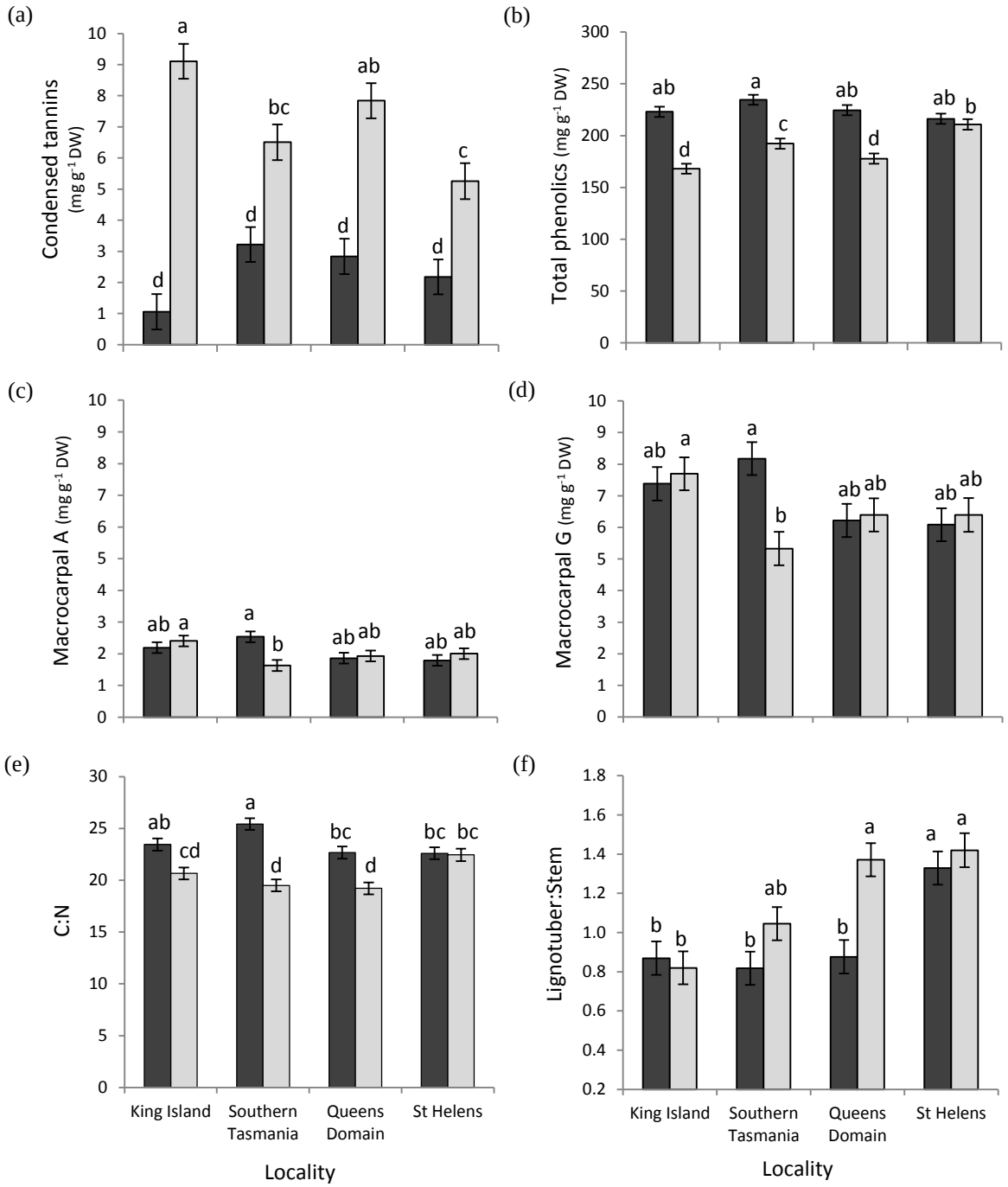

Figure 5. LSmean concentrations of (a) condensed tannins, (b) total phenolics, (c) macrocarpal A, (d) macrocarpal G, (e) carbon to nitrogen ratio (C:N) and (f) lignotuber to stem ratio in juvenile *Eucalyptus globulus* (dark bars) and *E. viminalis* (light bars) from four localities. Bars are standard error, and letters indicate significant difference ($P \leq 0.05$) after Tukey's *post hoc* test. Note different scales on y-axis among individual graphs. Condensed tannin results expressed as mg g^{-1} DW sorghum tannin equivalents. Total phenolic results expressed as mg g^{-1} DW gallic acid equivalents. Macrocarpals A and G expressed as mg g^{-1} DW macrocarpal A equivalents.

Leaf C:N and lignotuber size also varied differently between species at each location (species × locality interaction; Table 3). For example, *E. globulus* from KI, ST and QD contained a higher C:N than *E. viminalis* from each respective location, yet no significant difference in C:N was detected between species from SH (Fig. 5e). Lignotuber size also varied between species and among localities (Table 3). Lignotubers are used for carbohydrate storage and stem regeneration after disturbance (Whittock *et al.* 2003), and *E. viminalis* from drier sites (SH and QD) had larger lignotubers than *E. viminalis* from the wetter KI location (Fig. 5f). This pattern is somewhat also reflected in variation of *E. globulus* lignotuber size, with the largest lignotubers found on *E. globulus* from SH (dry) compared to QD (also dry), and the wetter ST and KI localities (Fig. 5f). Variation in lignotuber size between species from QD may reflect local site adaptations, as the *E. viminalis* at QD are situated on dry exposed slopes, whereas the *E. globulus* are situated in a nearby (c. 200 m) sheltered gully.

3.4.5 Effect of water limitation, recovery, and development on plant traits

The rarity of significant interactions of species or locality terms with the harvest or treatment terms in the mixed model (Table 3) indicate that for most traits the response to treatment and recovery was similar regardless of species or locality. Therefore, using results of the treatment × Harvest analysis (Table 3), we present the uniform effects of water deficit and recovery on plant traits regardless of plant provenance. First, traits varied between the controls at Harvest 1 and controls at Harvest 2, where the short period (two weeks) between harvests resulted in decreased LMA (Fig. 6a), CT (Fig. 6b) and TP (Fig. 6c) concentrations (Table 3). These trait changes are attributed to normal plant growth (Goodger *et al.* 2006; Loney *et al.* 2006b), and provide the base-line with which to compare the treatments over this period. For example, water limitation increased mean LMA and CT concentration above that of controls (Harvest 1), then recovery returned LMA and CTs to similar levels as controls (Harvest 2), even though LMA and CT concentrations decreased in

controls between the two harvests through normal plant development (Fig. 6a,b). Leaf water content decreased consistently with soil water deficit, however, unlike LMA and CT responses, the recovery period increased leaf water content slightly above that of controls at Harvest 2 (Fig. 6d). Responses of leaf C:N, chlorogenic acid and TP concentrations to water deficit and recovery were quite different from those described above. Water limitation did not impact leaf C:N or chlorogenic acid and TP concentrations, which remained quantitatively similar to controls at Harvest 1 (Fig. 6c, e-f). However, these traits did change once plants had been watered to field capacity for two weeks (Harvest 2), resulting in decreased leaf C:N (Fig. 6f) and concentrations of chlorogenic acid (Fig. 6e) and TPs (Fig. 6c) compared to controls.

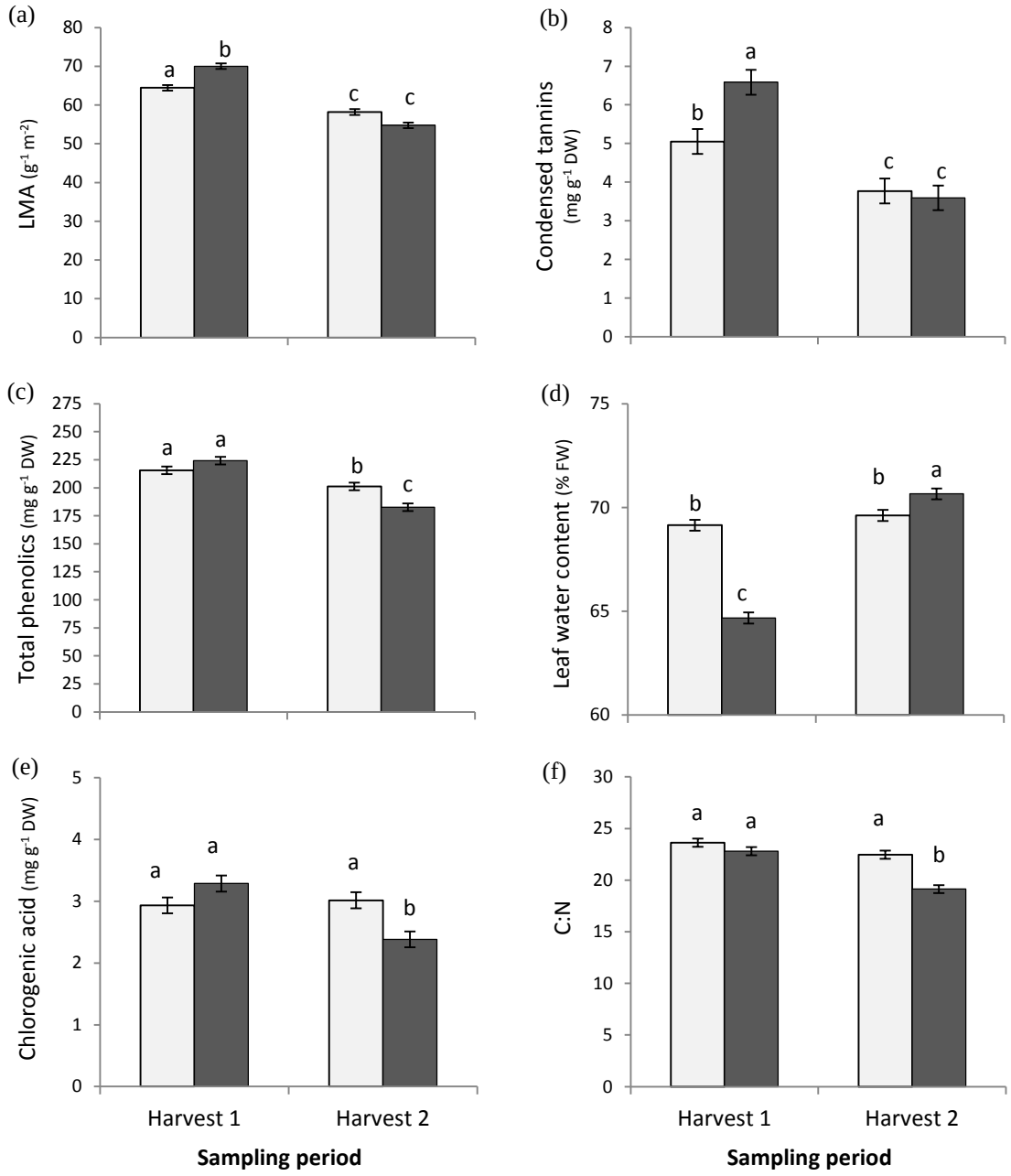

Figure 6. LSmean (a) leaf mass per area (LMA), (b) condensed tannins, (c) total phenolics, (d) leaf water content, (e) chlorogenic acid and (f) carbon to nitrogen ratio (C:N) of juvenile *Eucalyptus* (*E. globulus* and *E. viminalis*) leaves from plants grown under control (full water - open bars) and water limited (solid bars) treatments. Sampling period (x-axis) refers to plants harvested after 12 weeks (Harvest 1; control or water limited treatments) or after 14 weeks (Harvest 2; control or water limited and then recovered). Different individual plants were sampled at Harvest 1 compared to Harvest 2. Bars are standard error, and letters indicate significant difference ($P \leq 0.05$) after Tukey's *post hoc* test. Condensed tannin results expressed as mg g^{-1} DW sorghum tannin equivalents. Total phenolic results expressed as mg g^{-1} DW gallic acid equivalents. Leaf water content expressed as percentage fresh leaf mass (% FW). Note different scales on y-axes.

3.4.6 The effect of 12 weeks water limitation then 2 weeks recovery on plant traits

The full mixed model analysis revealed changes to a number of plant traits between treatments (Table 3). Treatment compared all control eucalypts (pooled Harvest 1 and Harvest 2 control data) against eucalypts that had altered water availability (pooled water limited [Harvest 1] and recovered [Harvest 2] plant data). The altered water treatment decreased mean plant biomass by 14% compared to the controls (Fig. 7a). The altered water treatment also had an impact on FPC concentrations, where concentrations of late-eluting FPCs were 7% lower (Fig. 7b), and mean macrocarpal A (Fig. 7c) and macrocarpal G (Fig. 7d) concentrations of altered watering treatment plants were 10-11% lower than in controls. Despite these changes, the levels of many PSMs were stable across treatments. Of note is the stability of all terpenes which were mainly affected by genetic factors, indicating low trait plasticity in response to this level of water deficit.

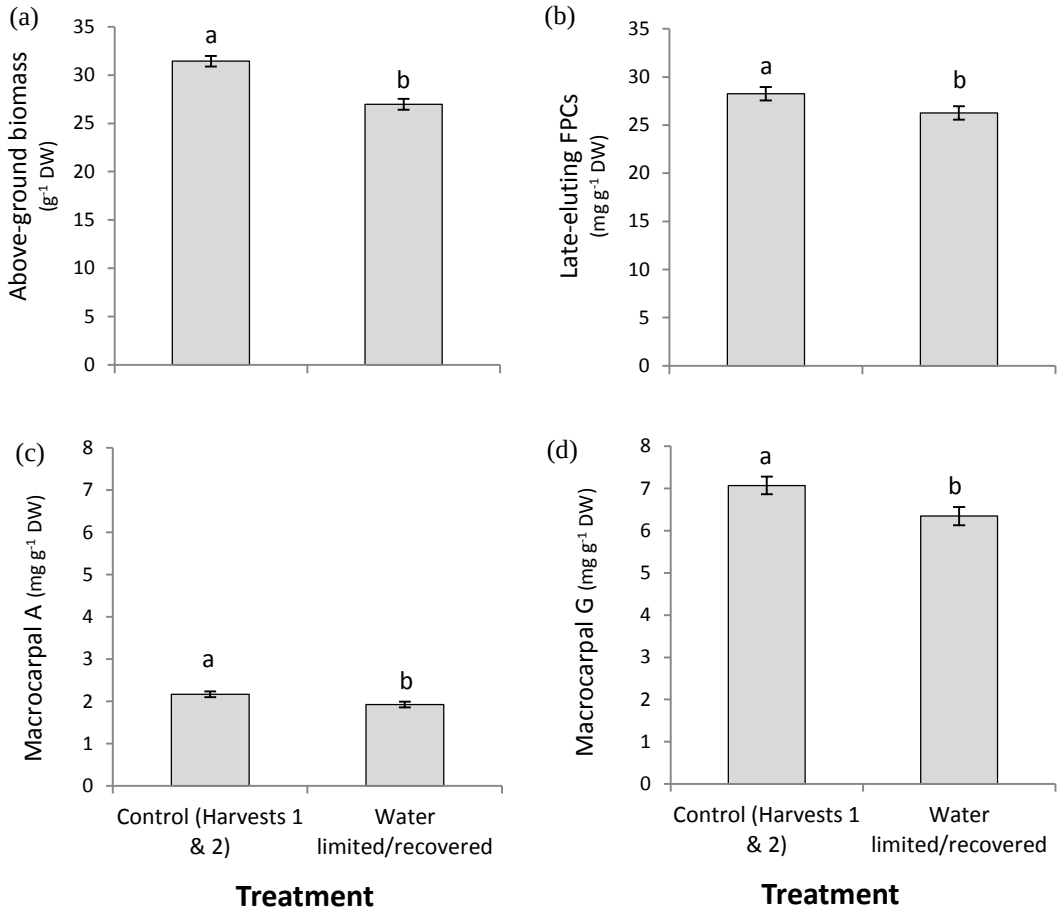

Figure 7. LSmean (a) above-ground biomass and concentrations of (b) late-eluting FPCs, (c) macrocarpal A and (d) macrocarpal G in juvenile eucalypts grown under control conditions (water daily to field capacity) throughout two sampling periods (Harvests 1 and 2) or altered water availability (plants grown under 50% water and sampled [Harvest 1] plus plants grown under 50% water then recovered for two weeks at field capacity [Harvest 2]). Bars are standard error, and letters indicate significant difference ($P \leq 0.05$) after Tukey's *post hoc* test. Note different scales on y-axis among individual graphs. Late-eluting FPCs and macrocarpal G expressed as mg g^{-1} DW macrocarpal A equivalents.

3.4.7 Trait variation among families within localities

Concentrations of the three FPCs varied significantly among families within localities regardless of species, whereas no significant intra-locality family variation was detected for any morphological or non-FPC chemical trait (Supplemental Table S5). This FPC variation among families (seed from a single mother tree) within localities was not affected by treatment or harvest main effects, or an interaction of these terms (Supplemental Table S5).

3.5 Discussion

We expected that the effect of water deficit on both species from wet localities would be larger than the effect on eucalypts from dry localities based on adaptation to local rainfall patterns and variation in mature *E. globulus* drought tolerance among localities (Dutkowski & Potts 2012). Responses to water deficit and recovery (increased, decreased and stable traits) were largely common across species and localities for all quantified morphological, physiological and PSM traits, even though variation in genetic-based plant traits was evident at both levels. The only exception was the effect of water deficit on foliar ABA levels which varied among localities, suggesting variation in how eucalypts from ST and SH 'perceived' the uniform amount of provided water (50%) compared to the other localities. Juvenile *Eucalyptus* from ST (wet) may have exploited the limited and uniform amount of water faster than eucalypts from other localities, and then used ABA to reduce stomatal aperture (Brodribb *et al.* 2014). However, this strategy was not evident in eucalypts from KI (also wet), while we did detect this type of response in eucalypts from a drier locality (SH), suggesting that induction of high foliar ABA levels is not simply related to rainfall patterns. Unfortunately, we did not quantify stomatal conductance, and neither Ψ_{leaf} or leaf water content differed among localities during water deficit (no locality × treatment interactions). As such, we were unable to define the strategy that eucalypts from ST and SH employed when experiencing water deficit. Interestingly, adult *E. globulus* from KI are highly drought susceptible

(Dutkowski & Potts 2012) and we found that juveniles of both species from KI made no attempt to reduce stomatal aperture during moderate soil water deficit (using ABA). Juvenile eucalypts from KI also have low constitutive levels of chlorogenic acid (antioxidant) and small lignotubers (e.g. used for carbohydrate storage and regeneration), and taken together with the limited plasticity of foliar ABA, may relate to the low drought adaptation of KI eucalypts. The similarity of these traits in both eucalypt species on KI compared to other localities is evidence of parallel evolution on this isolated island.

3.5.1 Genetically determined variation in traits between species and localities

Morphological and chemical traits could be divided into three groups based on patterns of genetic-based constitutive variation. These patterns are: (1) traits that differed among localities and the pattern was consistent in both species (locality effect and no species × locality interaction; Fig. 4); (2) traits that differed between species but showed little variation among localities (species effect and no species × locality interaction) and; (3) traits that varied among localities but the pattern of variation was species-specific (species × locality interaction; Fig. 5). Consistent patterns of trait variation among localities in two species (pattern 1) is excellent evidence of parallel evolution in response to selection pressures which differ among localities (McLean *et al.* 2014), leading to analogous population divergence in both species (O'Reilly-Wapstra *et al.* 2010). Parallel diversifying selection appears to be mainly influencing terpene concentrations in these species (Fig. 4). Wet localities have higher foliar terpene concentrations than dry locations, perhaps as defence against fungus/pathogens in wet environments (Ribera & Zuñiga 2012). Localised selection pressures may be acting on an individual terpene, where other terpenes are being indirectly selected due to genetic linkage (Kebede *et al.* 2012), or else the selection is for all these terpenes or a common precursor (Keszei *et al.* 2010; O'Reilly-Wapstra *et al.* 2010).

The second pattern of trait variation (pattern 2) described quantitative differences between the two *Eucalyptus* species, yet little or no variation among localities, alluding to conserved traits across each species range. Above-ground biomass, LMA, aromadendrene and globulol concentrations differed between species but not localities (and no species × locality interaction; Table 3), and so these traits may not have adaptive benefits at these localities. In contrast, macrocarpal (A and G) and phenol (TPs and CTs) concentrations differed among localities and the pattern was dissimilar between species (pattern 3; Fig. 5). Although macrocarpal concentrations varied between species and localities the pattern was similar for both compounds, a result of the close biosynthetic links between the similar macrocarpal isomers (Pass *et al.* 1998). While CT concentrations were greatest in *E. viminalis* and TP concentrations were greatest in *E. globulus* at most localities, TP concentrations did not differ between the *Eucalyptus* species at SH (Fig. 5b). High TP concentrations (or high concentrations of an unknown phenolic compound) in both species at SH may provide a localised fitness advantage at SH not required at the other localities, perhaps relating to local pathogen (Leser & Treutter 2005), herbivory pressures (Glynn *et al.* 2004) or antioxidant requirements (Živković *et al.* 2010), as SH is the most drought tolerant locality (Table 1).

3.5.2 Trait responses to water deficit differed depending on trait type

Water deficit altered traits associated with water stress (Ψ_{leaf} and foliar ABA level), decreased above-ground biomass and leaf water content as expected (Li *et al.* 2000), and increased LMA as shown in some other eucalypts (Ayub *et al.* 2011). More uncertain was the influence that water deficit would have on leaf PSM concentrations, and we found that responses were compound-specific, which supports the hypothesis proposed in Chapter 2 and tested here. Concentrations of FPCs (macrocarpals A and G, late-eluting FPCs) decreased during water deficit, CT concentrations increased, and the C:N and concentrations of all terpenes, TP and chlorogenic acid were not affected by the water deficit treatment. We know that

groups of like compounds share common biosynthetic pathways, and may be produced by the same multi-product enzymes (Mahmoud & Croteau 2002). For example, α-pinene, 1,8-cineole and limonene (among other monoterpenes) have geranyl diphosphate as a common precursor, whereas sesquiterpenes (and triterpenes) are formed from farnesyl diphosphate (Mahmoud & Croteau 2002; Keszei *et al.* 2010). We also know that macrocarpals share a common biosynthetic pathway (Pass *et al.* 1998), and so consistent responses to water deficit of compounds with biosynthetic links is expected as opposed to independent responses of each compound. As such, we show that related compounds in *Eucalyptus* respond similarly to soil water deficit, yet the level of trait plasticity and the direction of response (increased or decreased concentrations) varies among compound groups.

Changes to FPC (decreased) and CT (increased) concentrations evident in juveniles of these species during drought periods may flow on to affect the local community if similar responses occur in adult trees. For example, FPCs influence herbivore tree use and browsing patterns (Moore & Foley 2005; O'Reilly-Wapstra *et al.* 2010; Matsuki *et al.* 2011), and have antifungal (Lau *et al.* 2010; Tian *et al.* 2014) or antibacterial properties (Nagata *et al.* 2006), while tannins impact soil microbes (Ushio *et al.* 2013), invertebrate midgut bacterial communities (Mason *et al.* 2015) and mammal food intake (DeGabriel *et al.* 2009b). However, given the relatively modest changes to CT and FPC concentrations of juveniles during water deficit, the direct effect of these changes in juvenile eucalypts on plant-mediated interactions such as herbivory may be equally modest. The effect of water deficit on CT and FPC concentrations in adult *E. globulus* and *E. viminalis* from these locations is unknown, as is the flow on ecological effects.

3.5.3 Quantitative trait changes due to recovery from water deficit

Recovery from water deficit (re-watering) had two different effects on *Eucalyptus* leaf chemistry. First, traits that changed quantitatively due to water deficit (increased CT concentration) returned to control levels once recovered, demonstrating relatively rapid (2 weeks) plastic responses to water availability. Second, recovery influenced the concentrations of chemical traits not altered by water deficit. Specifically, concentrations of TPs, chlorogenic acid and the C:N decreased during recovery, even though carbon assimilation rates of eucalypts are overcompensated during recovery (increase above control plant assimilation rates; Correia *et al.* 2014). Decreased leaf C:N during recovery resulted from increasing leaf N content. High leaf N may provide a valuable source of nutrition to herbivores after natural drought periods (McArthur *et al.* 2003), depending on the protein binding action of concurrent increases to tannin concentrations in those same leaves. The observed decreases to chlorogenic acid and TP concentrations are linked, as chlorogenic acid is included as a small proportion (~1.5%) of the TPs. Phenols are natural antioxidants, and are utilised during cell rehydration to limit cell damage (Živković *et al.* 2010). As water deficit increases, levels of reactive oxygen species (ROS) in plant cells also increase, damaging cell components and causing oxidative stress (Beckett *et al.* 2012; Voss *et al.* 2013). This oxidative stress increases further at the beginning of plant recovery as plant metabolism resumes, maximising ROS abundance (Živković *et al.* 2010). Given the antioxidant properties of phenols, the decrease in TP concentrations (including chlorogenic acid) during plant recovery may represent the reduction of phenolic compounds while scavenging ROS.

3.5.4 Conclusions

Periods of drought are predicted to become more frequent and prolonged in many regions (Collins *et al.* 2013). We hypothesised that juveniles from genetically differentiated provenances of two species from higher rainfall localities would be more susceptible to soil water deficit, would exhibit greater water stress, and that

plant traits would be altered to a greater extent than in those plants from drier localities. We found that while quantitative variation in many constitutive traits among provenances suggests localised adaptation, induced responses of juveniles are still largely conserved among provenances of these two species, with the only evidence of intra-specific trait plasticity being the elevation of foliar ABA levels as an indication of stomatal adjustment. The overall uniformity of provenance level responses to soil water deficit within juveniles of these two *Eucalyptus* species would simplify predicting drought impacts on these species if the same response uniformity was to be found in juvenile and mature trees growing naturally at each locality.

Chapter 4

Trait changes in juvenile eucalypt leaves due to ontogeny, soil water deficit and recovery, and links to leaf flammability

Chapter 4 has been reviewed:

McKiernan AB, Hovenden MJ, Brodribb TJ, Potts BM, Davies NW, Rodemann T, O'Reilly-Wapstra JM. Trait changes in juvenile eucalypt leaves due to ontogeny, soil water deficit and recovery: Links to leaf flammability. *Annals of Botany*

4.1 Abstract

Soil water deficit can impact plant growth and alter chemical traits including plant secondary metabolites (PSMs). Here, variable durations of water deficit and a subsequent recovery period were used to quantify the effects on normal plant development and chemical traits linked to plant defence and flammability. Juvenile *Eucalyptus viminalis* of multiple provenances were grown under fully watered, water limited (provided 50% of control evapotranspiration), or water limited then rewatered (12 d full water) treatments, and plants were destructively sampled every 6 d for a maximum of 54 d. Leaf water potential (Ψ_{leaf}), foliar ABA level, growth, C, N, terpene, total oil yield, total phenolic, condensed tannin, chlorogenic acid and formylated phloroglucinol compound (FPC) concentrations were quantified. The flammability of a subset of samples was compared with leaf PSM levels. Water deficit caused minimal water stress, with changes to leaf water content and Ψ_{leaf} but not growth or foliar ABA levels. Regardless, water deficit increased leaf nitrogen, 1,8-cineole, α-pinene concentrations and the total oil yield, while decreasing C:N. Reduced available water did not affect concentrations of FPCs or phenolics. The 12 d recovery period largely reversed the changes induced by soil water deficit. Many traits changed over the 54 d period irrespective of water availability. High total oil yield was associated with a low spontaneous ignition temperature of leaf samples. Juvenile *E. viminalis* were able to maintain normal plant function and development when provided 50% water, with little water stress evident. Overall, *E. viminalis* provenance and development exerted a greater influence on PSM concentrations than water availability. While this water deficit treatment did affect concentrations of certain PSMs, severe water deficit causing water stress may have an even greater influence on PSM concentrations.

4.2 Introduction

Water is a fundamental resource required for plant growth and development (Bréda *et al.* 2006). Plants have evolved strategies and mechanisms to inhibit stomatal water loss and endure soil water deficit, while still accumulating atmospheric carbon dioxide (Brodribb & Cochard 2009). For example, stomatal closure and changes to cellular osmotic gradients aid conservation of within-plant water (Chaves *et al.* 2003), yet the duration of soil water deficit may vary the effectiveness of plant water conservation strategies. For example, short-term water deficit (days) may have minimal impact on plants as hormonal (abscisic acid [ABA]) regulation of stomatal conductance and within-plant osmotic gradients may provide short-term plant tolerance (Chaves *et al.* 2003). In contrast, toleration of long-term soil water deficit (weeks – months) may require pre-existing structural and physiological adaptations including reinforced vascular cells (Hacke & Sperry 2001; Blackman *et al.* 2010) and advanced stomatal control (Tardieu & Davies 1992; McDowell *et al.* 2008; Brodribb *et al.* 2014). Long-term water deficit may also influence the recovery potential of plants (Blackman *et al.* 2009).

The ability of plants to recover after water deficit depends on levels of hydraulic dysfunction or cavitation (Blackman *et al.* 2009; Brodribb & Cochard 2009; Cochard & Delzon 2013), and the speed at which conduction can be resumed (Martorell *et al.* 2014). Plant recovery ability varies between species (Galle *et al.* 2011) and also within species (Correia *et al.* 2014), and may differ depending on water stress duration (McDowell *et al.* 2008). The impacts of both soil water deficit (Bréda *et al.* 2006; McDowell *et al.* 2008; Mokotedi 2010; Pretzsch *et al.* 2013) and recovery (Aroca *et al.* 2008; Blackman *et al.* 2009; Galle *et al.* 2011; Correia *et al.* 2014; Martorell *et al.* 2014) on physiological and morphological traits have been investigated across a range of species. However, water deficit and recovery impacts on plant chemical traits have received less attention (Doran & Bell 1994; King *et al.* 2004; Leicach *et al.* 2010).

Plant secondary metabolites (PSMs) are chemicals which have diverse ecological impacts such as influencing plant decomposition, flammability and defence (Wiggins *et al.* 2006a; Ormeño *et al.* 2009; Youngentob *et al.* 2011; Chomel *et al.* 2014). As such, changes to PSM concentrations during water deficit or recovery can have flow-on consequences outside the plant (Gleadow & Woodrow 2002; Zhang *et al.* 2012). For instance, the total oil yield and individual oil components in plant foliage are linked to plant flammability (Ormeño *et al.* 2009; Steinbauer 2010) and defence against herbivory (Lawler *et al.* 1999b; Wiggins *et al.* 2003; Paine *et al.* 2011). The impact of soil water deficit on PSM concentrations are often compound-, species- and experiment-specific (King *et al.* 2004; Llusià *et al.* 2006; Llusià *et al.* 2010), and so general response trends have many exceptions (Herms & Mattson 1992; Pizarro & Bisigato 2010). Given the variability of water deficit impacts on PSM accumulation, the limited research involving PSMs and plant recovery (Marchese *et al.* 2010) and the previous focus on northern hemisphere tree species (Llusià & Peñuelas 1998; Turtola *et al.* 2003; Blanch *et al.* 2009), focussing on responses to water deficit in other dominant species may be of great benefit.

Eucalyptus is a dominant tree genus, and PSMs vary qualitatively and quantitatively among *Eucalyptus* species (Li *et al.* 1996; Nicolle *et al.* 1998; Steinbauer 2010). The concentration of PSMs in *Eucalyptus* leaves also vary within species, often between geographically and genetically separated provenances (O'Reilly-Wapstra *et al.* 2010; Andrew *et al.* 2013). *Eucalyptus* foliage contains a wide range of PSMs, including the non-nitrogen containing phenolic (including tannins) and terpene PSM groups, as well as specifically targeted PSM classes such as the formylated phloroglucinol compounds (FPCs) which influence mammal browsing (Wiggins *et al.* 2006a). As constitutive PSM concentrations vary among eucalypt provenances, the plasticity of these PSM levels in response to water deficit may also vary among provenances. A better understanding of the interaction between

soil water deficit, water deficit duration, plant recovery and PSMs among provenances within eucalypt species is needed.

Here, we utilised *Eucalyptus viminalis* seed from four provenances in a glasshouse-based trial, to investigate the impact of soil water deficit duration and subsequent recovery on normal plant development, by quantifying multiple plant traits. The four provenances were selected based on maximising geographic and environmental variation in order to assess intra-specific responses to treatments. The locations where provenances were sampled ranged from across a natural rainfall gradient. We hypothesised that responses to soil water deficit would be more evident in provenances from wet locations which may be less adapted to natural soil water deficit. We also hypothesised that 12 d water deficit would have minimal impact on plant traits (growth, PSMs, primary chemistry) compared to 66 d water deficit which would decrease overall carbon assimilation (McDowell *et al.* 2008), inhibiting growth and PSM accumulation. We incorporated a recovery treatment, where a subset of water limited plants were fully re-watered for 12 d prior to sampling, to assess the plasticity of morphological and chemical traits when re-watered. Finally, we assessed leaf flammability traits (ignitability and combustibility) in relation to chemical properties (Alessio *et al.* 2008a; Ormeño *et al.* 2009) to understand possible ecological consequences of altered PSM concentrations in juvenile *E. viminalis*.

The specific aims of this study were to determine; 1) if soil water deficit impacted specific chemical traits of juvenile *Eucalyptus viminalis* plants from four different provenances, 2) if responses to water deficit vary depending on the duration of the water deficit, 3) if relief from soil water deficit (recovery) would impact plant traits and 4) if leaf select chemical traits were associated with flammability metrics.

4.3 Materials and Methods

4.3.1 Plant material

Eucalyptus viminalis seed were collected from ten trees at each of three locations around Tasmania, and from King Island (KI), Australia (Chapter 3; Fig. 1 and Table 1). Open-pollinated seed obtained from a single mother tree was pooled and termed a 'family'. Mother trees were chosen from across each location, and were at least 100 metres apart to limit sampling from highly related individuals and to ensure genetic variation representative of the provenance. Mother trees on KI were spread across the island.

Eucalyptus viminalis seed from all mother trees (n = 40) were germinated and grown for one month. Uniform size (cotyledons and one leaf pair) seedlings were selected (>six per mother) and transplanted into individual plastic pots (base 38 x 38 mm, top 50 x 50 mm, height 118 mm). Potting mix contained eight parts composted fine pine bark: three parts coarse river sand, and N:P:K [19: 2.6: 10] at 1 g^{-1}/L potting mix. The pH was adjusted to approximately 6.0 with the addition of dolomite lime at 3 kg/m^{-3}. Eucalypts were grown for 12 weeks, then re-potted into larger pots (base 115 mm diameter, top 138 mm diameter, height 250 mm) containing exactly 900 g of potting mix (430 g^{-1} dry mass) with the addition of extra fertilizer (5 g Osmocote® 3-4 month [N14:P6.1 :K11.6] per pot) and wetting agent (Everris Hydraflo® at 1.35 L/m^{-2} potting mix). All 272 pots contained equal bulk density of potting mix and a single eucalypt. Potted plants were arranged in a randomised block design within a single naturally lit glasshouse (midday light c.1120 µmol m^{-2} s^{-1}; midday/night temperature 23/16°C [±2.6/2.2°C]), where plants of each locality (randomly selected with respect to family) were randomly but evenly divided between treatments, sampling periods, and four replicate blocks (two juvenile *E. viminalis* [one from each of two different random families of a locality] × four localities × 12 sampling times × three treatments – no recovery on days 0 and 6 [16 plants] = 272 plants). Plants of each locality, treatment and sampling time were

completely randomised within each replicate, watered daily, and grown for a further six weeks before experimental treatments were applied.

4.3.2 Experimental treatments

Treatments began in January 2013 (summer) when eucalypts were six months old, and followed methods outlined by Mitchell *et al*. (2013). One of three different watering regimes (treatments) was allocated to plants. Treatments were control (fully watered to field capacity daily), water limited (50% of mean evapotranspiration from controls, applied every 2 d) and recovery (as per water limited, but with 12 d watering to field capacity prior to harvesting). First, water was withheld from all plants in the water limited and recovery groups (controls continued to be watered) for 3 d to reduce pot water content and cause plant stress. After 3 d, half of these un-watered plants showed signs of turgor loss as indicated by wilting. At this time, the first batch of plants was harvested (Day 0 – control and water limited). Then, remaining un-watered plants were provided a reduced amount of water, calculated from control evapotranspiration over the previous 3 d period. Evapotranspiration was monitored daily (gravimetrically) on six control plants (mean daily evapotranspiration throughout experiment = 127 mL [±30 mL]), and 50% of the rolling daily mean evapotranspiration was provided to water limited plants every second day. The control pots used for determination of evapotranspiration were selected randomly and were re-selected weekly.

Watering continued throughout the experiment, and plants were harvested every 6 d for a total of 66 d, providing 12 sampling events (0 – 66 d). Six days after the first sampling (Day 0), the second sampling took place (Day 6). Every 6 d, a new batch of water limited plants began 12 d of watering to field capacity before being harvested 12 d later as ‘recovered’. Sampling on Days 12 – 66 included all treatments (control, water limited, recovered) while on Day 0 and Day 6 no recovery

plants were available. Plants harvested at each sampling time were the same age. Due to the hydrophobic nature of dry potting mix, plastic bags were placed over the base of all pots receiving limited water to eliminate loss of applied water without inhibiting evaporation from the soil surface. No waterlogging occurred, as excess applied water was taken up by the soil soon after watering. Plastic bags were removed when plants began the 12 d recovery period as drainage of excess water was required. Watering treatments continued until the final plants were harvested on Day 66.

4.3.3 Sample collection

Each sampling day coincided with the day water limited plants were not watered (watered every 2 d), whereas control and recovered plants were watered to field capacity that morning. Juvenile plants of all treatments due to be harvested that day were placed in full sunlight for >30 min. A subset of plants were randomly selected *a priori* for measurement of leaf water potential (Ψ_{leaf}) in order to quantify the impact of water deficit on plant stress (Brodribb & McAdam 2011). On Day 66 only, a single fully-expanded juvenile leaf of each plant in the subset (six control + 10 water limited + five recovery = 21 plants total) was taken during full sunlight around midday (11.30am – 2.00pm) when irradiance was 1500-2000 µmol m^{-2} s^{-1}, and Ψ_{leaf} quantified using a Scholander pressure chamber (PMS, Albany, OR, USA). Throughout the experiment, fully-expanded juvenile leaves were taken from multiple positions on each plant (~eight leaves per plant) to quantify foliar abscisic acid levels (ABA; a phytohormone which signals stomatal closure during water limitation), which we used as a proxy for stomatal adjustment during water stress. Leaves for ABA analysis taken from an individual plant were considered to represent the sampled plant. Sampled fully-developed leaves from three plants (same treatment) were pooled (~3 g leaf total) and immediately immersed in liquid nitrogen, then stored at -70°C until ABA quantification. Three pooled ABA samples were collected

from each treatment (n = 3) at each sampling time between Day 12 and Day 66 (90 pooled samples total).

All plants were then harvested individually using the following method, with eight plants (two plants from different random families from each of four localities) harvested every 6 d from each treatment. The stem was cut and total fresh above-ground biomass was determined. As we were only interested in leaves which developed during the treatment period for chemistry, we harvested only the uppermost 50% of leaves (highest four nodes of main stem below apical bud), disregarding leaves which were not fully-expanded or showed signs of damage. Leaves were stripped from the stem, pooled and mixed. A random sub-sample was frozen (-12°C) for oil analysis, then remaining leaves were weighed, frozen, freeze dried, re-weighed, scanned using near infrared spectroscopy (NIRS – see below), ground in a Cyclotec™ 1093 cyclone mill (Foss, Hillerød, DK) and passed through a 1 mm sieve for analysis of primary chemistry (Thermo Finnigan EA 1112 Series Flash Elemental Analyser, Italy), total phenolics, condensed tannins, chlorogenic acid, formylated phloroglucinol compounds and flammability (n = 7 samples for flammability). Sample mass prior to and post lyophilisation was used for quantification of fresh leaf water content (100-[dry mass/fresh mass ×100]), expressed as percentage fresh leaf weight (% FW). The fresh stem and unharvested leaves were weighed and oven dried at 40°C until constant mass. Total dry above-ground biomass was determined using dry stem and leaf mass, fresh total above-ground biomass, and the calculated dry mass of harvested leaves.

4.3.4 Abscisic acid (ABA)

A subset of pooled ABA samples (n = 20) were used to assess the impact of treatments on leaf ABA content (five control, 10 water limited and five recovery samples collected throughout the sampling period), with twice the number of water

limited samples assayed (n = 10) as this was where we expected to observe variation. Extraction of leaf ABA followed the method described by McAdam and Brodribb (2014) except that ~0.2 g of pooled leaves were used. Endogenous ABA was quantified using a Waters ultra-performance liquid chromatograph coupled to a Xevo triple quadrupole mass spectrometer (UPLC-MS), with results expressed as ng g^{-1} FW.

4.3.5 Quantification of secondary chemistry

Extraction, analysis, quantification and identification of total oil and oil components (1,8-cineole, α-pinene, globulol, aromadendrene and limonene) followed the method outlined by McKiernan *et al.* (2012). Concentrations of 1,8-cineole and α-pinene were expressed as mg g^{-1}DW (dry weight) using standards, while total oil and other oil components were expressed as mg g^{-1} DW cineole equivalents. Phenols were extracted, then total phenolics (TPs) and condensed tannins (CTs) were quantified in duplicate as described in Chapter 2 (section 2.3.4), with the exception that a VIS-7200A UV-visible spectrophotometer (Techcomp Limited, Hong Kong) was used. TP results were expressed as mg g^{-1}DW gallic acid equivalents, CT results were expressed as mg g^{-1}DW sorghum tannin equivalents. We assayed two specific FPCs (macrocarpals A and G) by High Performance Liquid Chromatography (HPLC) following the methods of Wallis and Foley (2005). A group of relatively late eluting FPCs (eluting just before and after macrocarpal G) were also quantified. Macrocarpal A was expressed as mg g^{-1}DW, while macrocarpal G and the group of late-eluting FPCs were expressed as mg g^{-1}DW macrocarpal A equivalents, using standards described by Eyles *et al.* (2003a). Chlorogenic acid was extracted and quantified as described in Chapter 3 (section 3.3.7). Near Infrared Spectroscopy (NIRS) and wet chemical values (from a subset of samples) were used to create predictive models, and then to predict chemical values of each trait in the remaining samples as described previously (section 3.3.8).

4.3.6 Flammability of leaf material

A subset (n = 7) of freeze-dried and ground leaf samples were used to investigate how leaf chemistry affected sample flammability. Dried and ground samples were used for the assay to eliminate the effects of leaf morphology and residual water content on flammability (Liodakis *et al.* 2008). Samples were selected to maximise variation of total oil yield and TP concentrations with the aim of correlating total oil yield, 1,8-cineole, α-pinene, TP and CT concentrations with flammability traits. Selected samples had a TP range of 140 – 375 mg g^{-1}DW (gallic acid equivalents) and a total oil yield range of 22 – 88 mg g^{-1} DW (cineole equivalents). These seven samples had previously been assayed in triplicate as described above for quantification of each chemical trait. Sample analysis was carried out using a TA Instruments SDT 2960 (New Castle, DE, USA) simultaneous differential temperature analyser coupled to a thermogravimetric analyser (DTA-TGA). For each sample, approximately 5 mg of sample was placed into a 110 µL platinum crucible (exact mass recorded) and heated to 600°C at 10°C per minute in an air atmosphere flowing at 100 ml/min (Liodakis *et al.* 2008). The temperature scale was calibrated using the melting points of indium (156.5985°C), tin (321.93°C), zinc (419.53°C) and silver (961.78°C), while the balance was calibrated using standard weights provided by the manufacturer. Simultaneous TGA and DTA data for each sample were analysed in Universal Analysis 2000 (TA Instruments, New Castle, DE, USA) using parameters outlined by Liodakis *et al*. (2008). Relative spontaneous ignition temperature (RSIT) is the temperature (°C) at which the onset of gas-phase combustion occurs (the first exothermic peak). Maximum weight loss rate (MWLR) was taken from each peak (expressed as percentage mass loss per degree temperature change [% $°C^{-1}$]) and is positively related to the combustion rate (Liodakis *et al.* 2008). Total combustion duration (CD) is the exothermic offset point minus the onset point (min).

4.3.7 Statistical analysis

All analyses were completed using SAS statistical software package (version 9.2, SAS Institute Inc., Cary USA). Testing of fixed effects (treatment, locality and duration) was undertaken using various general linear models (PROC GLM of SAS) depending upon the data set. As Ψ_{leaf} data was from limited plants, and ABA data was pooled samples rather than from a single plant, only treatment (control, water limited and recovery) was fitted in the model for these traits. For all other traits, treatment, locality (n = 4), duration (Day 12 to 66) and two way interactions were fitted. The three way interaction (duration × treatment × locality) was not included due to insufficient replication at that level. Data from Day 0 and Day 6 was excluded from the main analysis as only two treatments were available (control and water limited). However, these data were included in an analysis involving just control and water limited treatments to clarify changes to TP concentrations over time detected in the main analysis. Residuals for all variables were checked for assumptions of normality and heterogeneity of variances, and no transformations were necessary. Due to the number of individual analyses performed (n = 17), the false discovery rate was controlled following Benjamini and Hochberg (2000). The correlation amongst flammability traits (RSIT, MWLR for each peak, CD), total oil yield, α-pinene, 1,8-cineole, TP and CT concentrations were analysed using (PROC CORR of SAS). In order to summarise the multivariate patterns of chemical variation, a number of secondary chemical traits (macrocarpals A and G, TPs, CTs, chlorogenic acid, 1,8-cineole, α-pinene, limonene, aromadendrene and globulol) were analysed using discriminant analysis (PROC DISCRIM of SAS) with each duration × treatment and locality × treatment combination treated as a separate group. Low replication at the three-way interaction level made a single discriminant analysis unsuitable, and so these two-way interactions were analysed separately.

4.4 RESULTS

Significant variations in many traits were identified between the four localities and over the duration of the experimental sampling (Table 1). Significant differences in PSM concentrations were detected among treatments, even though few signs of water stress were found in water limited plants. No two-way interactions were found between any of the main effects after controlling the false discovery rate (Table 1), indicating that locality responses to the treatments were similar (no locality × treatment interaction) and that traits in juveniles of the four localities changed equally over time (no locality × duration interaction). One of our main hypotheses was that short-term water deficit would influence plant traits less than long-term water deficit; however, the treatment effects were consistent regardless of treatment duration (no treatment × duration interaction). Therefore, only results for each of the individual main effects (locality, treatment and duration) are presented below.

Table 1. Results of general linear model (GLM) analysis of juvenile *Eucalyptus viminalis* trait variation between localities (KI, ST, QD, SH), treatments (control, water limited, recovered), treatment durations (9 × 6 day intervals) and two-way interactions[1]

	Locality		**Treatment**		**Duration**		**Locality x Treatment**		**Locality x Duration**		**Treatment x Duration**	
	$F_{3,248}$	***P***	$F_{2,248}$	***P***	$F_{9,248}$	***P***	$F_{6,248}$	***P***	$F_{27,248}$	***P***	$F_{18,248}$	***P***
Above-ground biomass	0.5		2.1		14.1	***	2.5	#	1.9	#	1.0	
Leaf water content	8.2	***	7.4	***	7.6	***	2.5	#	1.2		1.1	
C:N[a]	3.9	*	8.2	***	10.2	***	1.3		1.1		1.0	
Carbon[a]	4.0	**	1.6		2.2	*	0.4		0.7		0.8	
Nitrogen[a]	4.9	**	7.0	**	9.4	***	1.4		1.1		0.8	
1,8-Cineole[b]	5.8	***	7.0	**	7.7	***	2.5	#	1.5		0.9	
α-Pinene[b]	5.6	**	4.8	**	3.0	**	2.2	#	0.9		1.1	
Aromadendrene[b]	2.7		2.4		0.9		0.5		0.8		0.8	
Globulol[b]	4.7	**	2.1		0.6		2.1		0.8		0.8	
Limonene[b]	13.7	***	3.1	#	4.7	***	1.1		1.2		0.6	
Total oil	14.8	***	4.9	**	2.5	*	0.9		0.6		0.5	
Chlorogenic acid[c]	8.3	***	0.7		4.8	***	0.9		0.9		1.4	
Condensed tannin[c]	5.4	**	0.9		16.6	***	1.8		1.4		1.7	#
Total phenolics	21.0	***	2.2		2.9	**	0.6		0.9		0.7	
Macrocarpal A[cd]	15.4	***	2.8		2.1	*	1.1		0.9		1.8	#
Macrocarpal G[cd]	11.7	***	4.0	#	1.4		1.1		0.8		1.5	
Late-eluting FPCs[cd]	17.1	***	2.4		1.5		1.1		1.0		1.5	

[1] *KI* King Island. *ST* Southern Tasmania. *QD* Queens Domain. *SH* St Helens

*indicates $P \leq 0.05$. ** indicates $P < 0.01$. *** indicates $P < 0.001$.

# indicates $P \leq 0.05$ but not significant after correction for false discovery rate (Benjamini & Hochberg 2000)

[a] indicates element, [b] indicates terpene within total oil, [c] indicates phenol within total phenolics, [d] indicates formylated phloroglucinol compound (FPC).

4.4.1 Trait variation among localities/provenances

All traits differed between the four localities except above-ground biomass and the concentration of aromadendrene (Table 1). For example, concentrations of individual terpenes (1,8-cineole, α-pinene, globulol and limonene) and the total oil yield were slightly higher in *E. viminalis* from the wet KI locality, whereas concentrations were generally lower in leaves from the dry SH locality, or SH in the case of globulol (Table 2). In contrast, FPC concentrations (macrocarpals A and G, and late-eluting FPCs) appeared to group latitudinally rather than with rainfall, as FPC concentrations were greater in the low latitude KI and SH compared to the higher latitude ST and QD locations (Table 2). Also, the variation in concentrations of terpenes (10-23%) was less than variation in FPC concentrations between localities (26-35%). Patterns of phenol concentrations were more varied between localities (Table 2). Chlorogenic acid concentrations were generally greater in low latitude (ST, QD) compared to high latitude localities (KI, SH), yet CT concentrations were higher in wet (KI, ST) compared to dry (QD, SH) localities (Table 2). Interestingly, the pattern of TP concentration was different to any other trait, with the highest concentrations in *E. viminalis* from ST and SH, and lower in plants from KI and QD (Table 2). Levels of phenol variation among localities were similar the levels of FPC variation (26-35%).

Table 2. Least squares mean (LSmean) concentrations (±SE) of chemical traits in juvenile *Eucalyptus viminalis* leaves from four localities[2]

	Locality			
Trait	KI	ST	QD	SH
C:N	43.1 (0.8)^{ab}	43.8 (0.9)^{ab}	40.5 (1.2)^{b}	46.3 (1.2)^{a}
1,8-cineole^{x}	23.1 (0.3)^{a}	22.9 (0.4)^{a}	21.5 (0.5)^{ab}	20.9 (0.5)^{b}
α-pinene^{x}	5.5 (0.1)^{a}	5.0 (0.2)^{ab}	4.7 (0.2)^{b}	4.7 (0.2)^{b}
Aromadendrene^{x}	2.9 (0.1)^{ns}	2.6 (0.1)^{ns}	2.8 (0.1)^{ns}	2.7 (0.1)^{ns}
Globulol^{x}	1.9 (0.0)^{a}	1.7 (0.0)^{b}	1.8 (0.1)^{ab}	1.8 (0.1)^{ab}
Limonene^{x}	2.6 (0.1)^{a}	2.3 (0.1)^{a}	2.1 (0.1)^{b}	2.0 (0.1)^{b}
Total oil^{x}	46.1 (0.6)^{a}	43.2 (0.7)^{b}	41.9 (1.0)^{bc}	38.6 (0.9)^{c}
Chlorogenic acid^{y}	4.2 (0.2)^{b}	5.7 (0.2)^{a}	5.0 (0.3)^{ab}	4.5 (0.3)^{b}
Condensed tannins^{y}	10.0 (0.6)^{a}	9.1 (0.7)^{ab}	8.0 (0.9)^{b}	7.6 (0.9)^{b}
Total phenolics^{y}	246.3 (3.1)^{b}	273.5 (3.5)^{a}	252.1 (4.5)^{b}	284.6 (4.5)^{a}
Macrocarpal A^{yz}	2.0 (0.1)^{a}	1.3 (0.1)^{b}	1.5 (0.1)^{b}	1.8 (0.1)^{a}
Macrocarpal G^{yz}	5.6 (0.2)^{a}	3.7 (0.3)^{c}	4.2 (0.3)^{bc}	4.9 (0.3)^{ab}
Late-eluting FPCs^{yz}	28.1 (0.7)^{a}	20.7 (0.8)^{b}	22.3 (1.1)^{b}	25.0 (1.1)^{a}

[2]Letters indicate significant difference ($P \leq 0.05$) between the four localities across all treatments and sampling times for each trait after Tukey's *post hoc* tests. Localities ranked in order of decreasing rainfall from *KI* King Island, *ST* Southern Tasmania, *QD* Queens Domain to *SH* St Helens. *C:N* carbon to nitrogen ratio. ^{x} indicates terpene, ^{y} indicates phenol, ^{z} indicates formylated phloroglucinol compound (FPC), ^{ns} non-significant ($P > 0.05$).

4.4.2 Trait variation due to treatments

Water deficit had no impact on plant growth (Table 1) or on mean foliar ABA levels (Fig. 1). Ψ_{leaf} varied between the three treatments ($F_{2, 118}$=52.55; P<0.0001; Fig. 1), as the Ψ_{leaf} of water limited eucalypts (-0.92 MPa) was more negative than control (-0.43 MPa) and recovery plants Ψ_{leaf} (-0.38 MPa; Fig. 1). Soil water deficit also affected leaf water content (Table 1), as water limited leaves (63% FW) contained less water than control and recovered plants (65% FW respectively; Fig. 2a). In summary, the level of water deficit applied was sufficient to slightly decrease Ψ_{leaf}, but not sufficient to impact the ability of these *E. viminalis* to grow or presumably to close stomata, as indicated by no significant increase in mean foliar ABA levels (Fig. 1). Therefore, the water deficit treatment did not cause noticeable plant water stress.

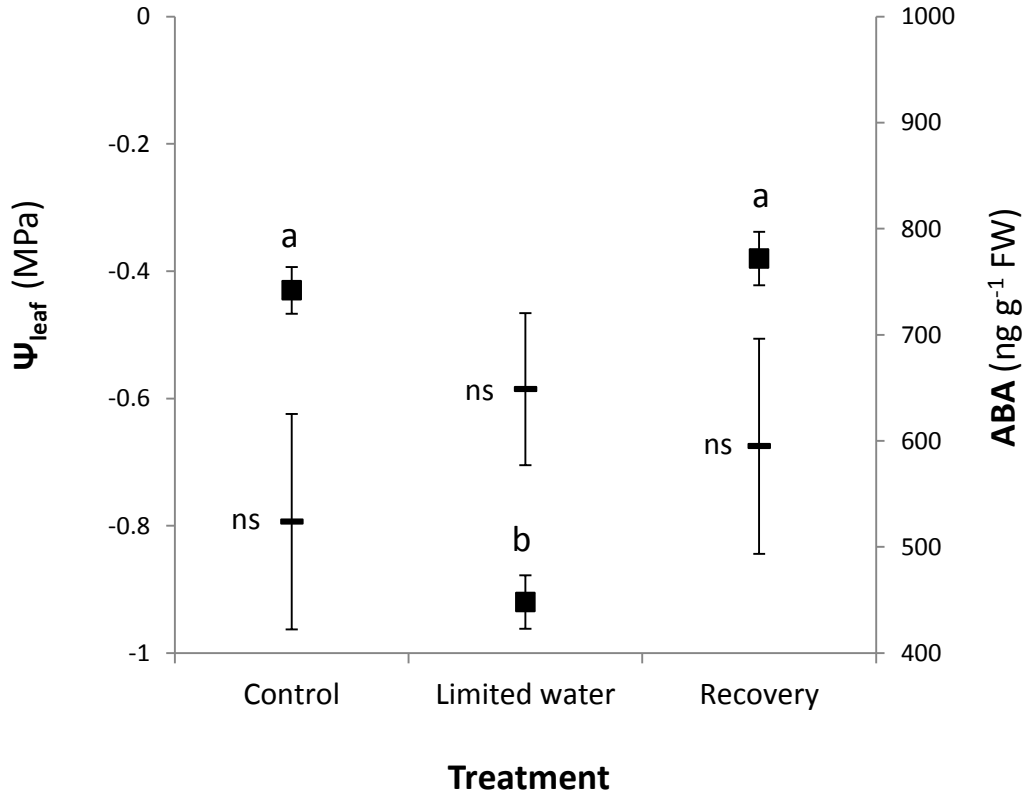

Figure 1. Least squares mean (LSmean) leaf water potential (Ψ_{leaf}; solid squares) and leaf abscisic acid (ABA; horizontal lines) of juvenile *Eucalyptus viminalis* grown under one of three watering regimes (control, limited water, recovery) in a glasshouse experiment. Water potential and ABA data analysed separately. Bars indicate standard error (±SE). Different letters above Ψ_{leaf} data points indicate significant difference ($P \leq 0.05$) after Tukey's *post hoc* tests. *ns* indicates no significant difference in leaf ABA content between treatments

Even though plant stress was virtually absent, water deficit and recovery treatments influenced many *E. viminalis* PSM and primary chemical traits (Table 1). Water limitation reduced leaf C:N below levels in control plants (41:1 and 46:1; respectively), and recovery (44:1) increased leaf C:N back to control plant levels (Fig. 2b). These C:N changes reflect water limitation increasing N by 11% compared to controls (Fig. 2c) while leaf C content remained stable (Table 1). The soil water deficit treatment also impacted the concentrations of total oil and the two major oil components, 1,8-cineole and α-pinene (Table 1). Water limitation increased mean 1,8-cineole concentration by 8% (Fig. 2d), α-pinene concentration by 11% (Fig. 2e), and total oil yield by 7% compared to controls (Fig. 2f). Juvenile *E. viminalis* that were re-watered for 12 d prior to harvesting (recovery treatment) had 1,8-cineole, α-pinene and total oil concentrations similar to controls, indicating that 12 d was enough time to reverse the impact of water deficit on terpene concentrations (Fig. 2d-f). Water deficit had no influence on the concentrations of FPCs (macrocarpals and late-eluting FPCs), phenolics (total phenolics, condensed tannins and chlorogenic acid), limonene, globulol or aromadendrene in these *E. viminalis* (Table 1).

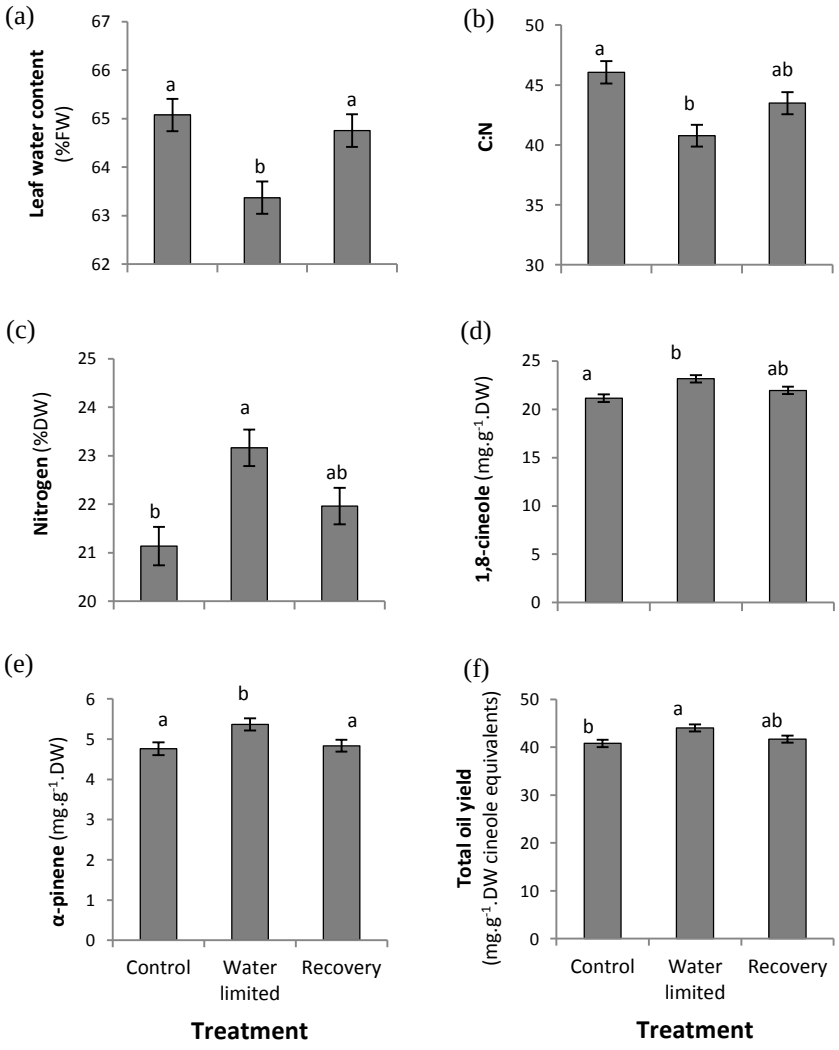

Figure 2. Least squares mean (LSmean) values of juvenile *Eucalyptus viminalis* traits between control (watered to field capacity), water limited (received 50% water of controls) and recovery (water limited and then 12 days water to field capacity) treatments. Error bars indicate standard error. Different letters within each graph indicates significant difference ($P \leq 0.05$) after Tukey's *post hoc* tests. Note scale variation on y-axis.

4.4.3 Trait variation over the duration of the experiment (plant development)

Many traits changed quantitatively over the duration of sampling (54 days), except for concentrations of two terpenes (aromadendrene and globulol), macrocarpal G and the late eluting FPCs which remained stable from Day 12 until Day 66 (Table 1). Overall, *E. viminalis* continued to grow during the sampling period, with average plant biomass ranging from 6.3 g^{-1} DW on Day 12 to 11.5 g^{-1} DW on Day 66. Mean leaf water content decreased during this time from 67% (FW) on Day 12 to 61% (FW) on Day 66, irrespective of treatment (i.e. no treatment × duration interaction; Table 1). Leaf C:N increased over time beginning on Day 12 at 35:1 and peaking at Day 66 with 51:1, due largely to a 32% decrease in leaf N over the 54 d (Fig. 3a), while overall leaf C content was relatively stable. Specifically, even though leaf carbon changed significantly over the sampling period (Table 1), *post-hoc* test found no significant differences in leaf carbon among individual sampling dates (Fig. 3b).

Concentrations of many terpenes changed over the sampling period (Table 1). Concentrations of 1,8-cineole increased from around 20 mg g^{-1}DW on Day 12 to around 24 mg g^{-1}DW by Day 66 (Fig. 3c), yet changes to the concentrations of the other terpenes were less convincing following *post-hoc* tests. Firstly, the total oil yield generally increased up until Day 60 (from 40 mg g^{-1} DW up to 45 mg g^{-1} DW cineole equivalents), yet total oil concentrations of Day 66 (42 mg g^{-1} DW cineole equivalents) were lower than expected following the trend, and *post-hoc* tests identified no differences among individual sampling days (Fig. 3f). Likewise, *post-hoc* tests failed to identify significant variation in α-pinene concentrations among sampling days (Fig. 3d). *Post-hoc* tests did provide support for an effect of duration on limonene concentrations (Fig. 3e). While limonene concentrations of Day 12 differed to many other sampling days, the trend suggests relatively stable concentrations overall, and that the low concentration of Day 12 (1.7 mg g^{-1} DW cineole equivalents) may be disproportionately influencing the results (Fig. 3e).

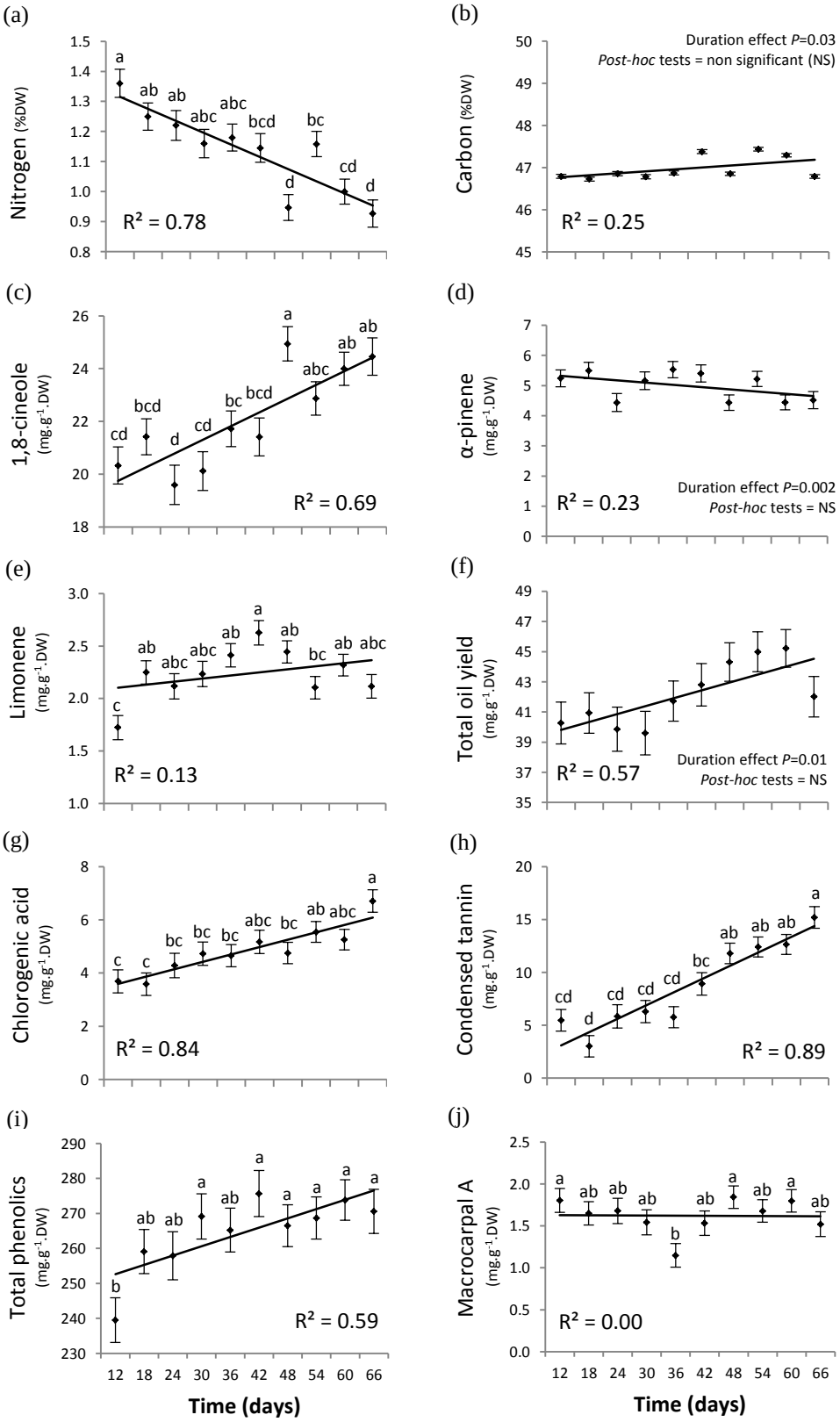

Figure 3. Least squares mean (LSmean) values of juvenile *Eucalyptus viminalis* traits between sampling times. No significant treatment effect was found to influence trait changes over time (no treatment × duration interaction; Table 1), so each data points represent control, water limited and recovery plant data. Error bars indicate standard error. Note scale variation on y-axis. Trend-lines are linear regression to indicate overall trends.

Concentrations of phenolics accumulated over time with normal plant development (Table 1), and the increase was more certain than seen in the terpenes. Chlorogenic acid concentration increased almost two-fold from 3.6 mg g^{-1} DW on Day 12 up to 6.7 mg g^{-1} DW on Day 66 (Fig. 3g), while CT concentrations increased four-fold over the sampling time (Day 18 = 3 mg g^{-1} DW and Day 66 = 15.2 mg g^{-1} DW; Fig. 3h). Total phenolic concentrations also increased over the experimental duration (Table 1), beginning on Day 12 at 239.5 (mg g^{-1}DW) and increasing by 11% to 270.6 (mg g^{-1}DW) by Day 66 (Fig. 3i). *Post-hoc* tests showed that TP differences between sampling times (Table 1) relied on the low TP concentration on Day 12 (Fig. 3i). As such, previously excluded control and water limited data from Day 0 and Day 6 (no recovery data) was included in the analysis for trend clarification, made possible as treatment had no effect on TP concentrations (Table 1). Mean TP concentration of Day 0 and Day 6 plants were also low (226.8 and 253.8 mg g^{-1}DW, respectively), and so we conclude that TP concentrations did increase over time, at least to Day 42 (Fig. 3i). Of the FPCs, only macrocarpal A concentration was found to differ significantly between sampling periods (Table 1). However, changes to macrocarpal A concentrations over time did not form a clear trend, and relied on the low macrocarpal A content of Day 36 plants (1.1 mg g^{-1} DW; Fig. 3j). Therefore, as with most terpenes (all except 1,8-cineole) we conclude that all FPCs remained fairly stable during plant development.

4.4.4 Discriminant analysis and ordination using multiple chemical traits

The multivariate analysis investigated differences between the impact of treatments over the duration of the experiment (Fig. 4a) and the variation of treatment impacts on the four localities (Fig. 4b). The resulting multivariate plots showed that the main difference in the overall PSM profile of leaves was due to changing PSM concentrations over the sampling period (Fig. 4a). Specifically, the chemical trajectories of these PSMs changed around day 36 or 42 regardless of treatment (Fig. 4a), which may be linked to changes in limonene, TP and macrocarpal A patterns around this time (Fig. 3). The effect of water deficit was

clearly less than the effect of plant development (Fig. 4a) and that of the genetic-based constitutive differences between localities (Fig. 4b). Chemical properties of juvenile *E. viminalis* from KI appeared the most different of the localities, although the overall PSM profile of ST plants was also distinct from QD and SH (Fig. 4b). The two dry east-coast localities (QD and SH) had very similar patterns of chemical concentrations, whereas the chemical profile of wet localities (ST and KI) differed from the dry localities, and also differed from each other (Fig. 4b). These locality differences were generally maintained regardless of watering regime.

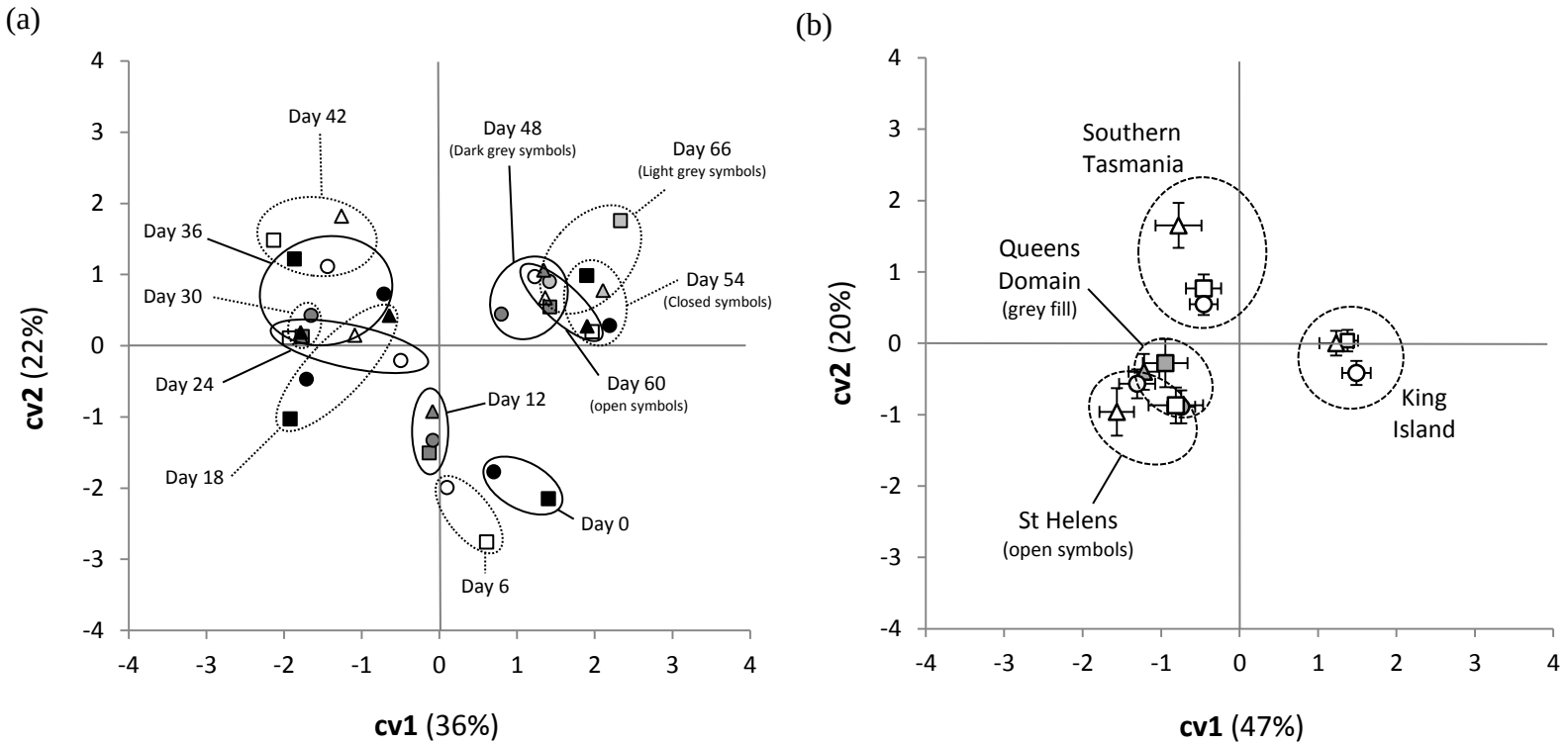

Figure 4. Ordinations summarising the overall response of (a) juvenile *Eucalyptus viminalis* leaf chemical traits to three watering treatments (control [squares], water limited [circles] and recovery [triangles]) over 12 sampling periods (0d - 66d at 6d intervals) and (b) four *E. viminalis* provenances to the three watering treatments. Symbol colour (open, closed, light grey, dark grey) and broken/unbroken lines have been used to aid interpretation of data with regard to sampling day (a) and provenance (b). Percentages along each axis indicate the proportion of variation explained by each canonical coefficient. The axes are the first two canonical variates derived from discriminant analysis. Bars on (b) indicate ±SE. The analysis was based on macrocarpal G, macrocarpal A, late-eluting FPCs, total phenolic, condensed tannin, chlorogenic acid, 1,8-cineole, α-pinene, limonene, aromadendrene, globulol and total oil yield data.

4.4.5 Flammability

Exothermic curves obtained using Differential Thermal Analysis (DTA) indicate the rate of mass loss from a sample of known mass (combustion) during a steady increase in temperature (Liodakis *et al.* 2008). As such, a peak in the curve indicates rapid mass loss at a given temperature (high combustion rate) and a trough in the curve indicates slow loss of mass at a temperature (low combustion rate). Four exothermic peaks were identified in these seven freeze-dried and ground samples, with the most variation between samples relating to peak 3 and peak 4 (Fig. 5). Peaks occurring in lower temperatures (~ 300°C) are associated with gas-phase combustion and higher temperatures (~ 400°C) can relate to solid-phase combustion of leaves (Liodakis *et al.* 2008). Here, the first exothermic peak maximised at around 240°C, the second around 320°C, the third around 430°C, and the final and most variable exothermic event (peak 4) occurred around 480°C depending on the sample (Fig. 5).

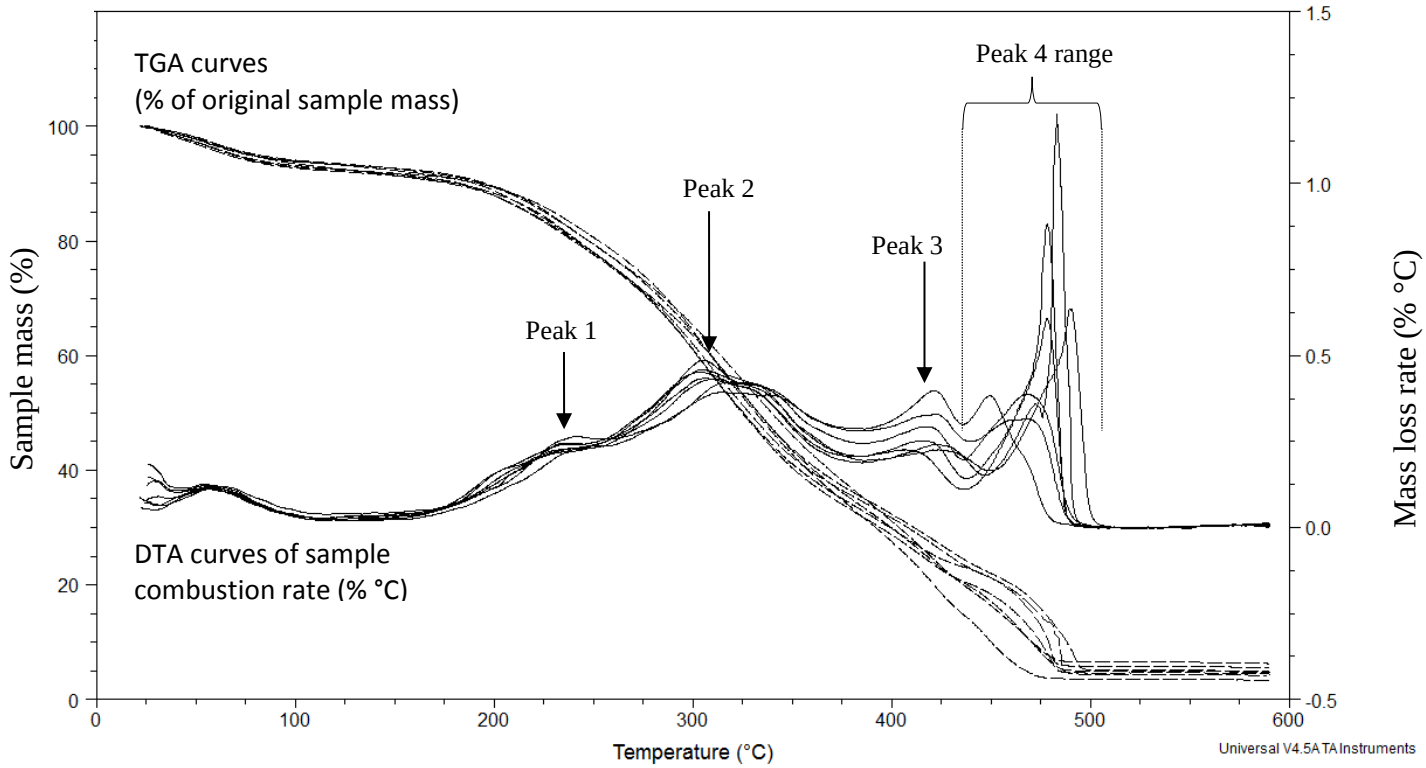

Figure 5. Thermogravimetric Analysis (TGA) and Differential Thermal Analysis (DTA) curves of ground juvenile *Eucalyptus viminalis* leaf samples (n=7) in air. TGA curves (broken lines) show change in mass of dried and ground leaf sample (% of original sample mass) in relation to increasing temperature (10°C per minute in an air atmosphere flowing at 100 ml/min). DTA curves (solid lines) show the sample mass loss rate over the temperature range (expressed as % °C). Four exothermic peaks were identified on the DTA curves, indicating periods of rapid sample combustion. Arrows indicate maximums of exothermic peaks 1-3. Peak four maximums were highly variable between samples, and so the Peak 4 range is indicated within dotted lines.

In the assayed samples, the variation in flammability traits was significantly correlated in several cases (Table 3). These included negative correlations between the relative spontaneous ignition temperature and combustion duration, between combustion duration and the maximum peak 3 combustion rates, and a positive correlation between combustion duration and the peak 3 combustion rates (Table 3). These correlations mean that the samples which combusted at lower temperatures also combusted for longer, and had a lower maximum combustion rate at c. 430°C.

Table 3. Pearsons correlation coefficients among *Eucalyptus viminalis* chemical and flammability traits from simultaneous TGA/DTA analysis of freeze-dried and ground juvenile leaves (n=7)[3]

	TPs	CTs	Oil	1,8-cineole	α-pinene	RSIT	$MWLR_1$	$MWLR_2$	$MWLR_3$	$MWLR_4$
CT	0.44 ns									
Total oil yield	**-0.83** *	-0.42 ns								
1,8-cineole	**-0.78** *	-0.40 ns	**0.99** ***							
α-pinene	-0.72 ns	0.01 ns	0.56 ns	0.49 ns						
RSIT	0.68 ns	0.51 ns	**-0.84** *	**-0.83** *	-0.67 ns					
$MWLR_1$	0.57 ns	-0.35 ns	-0.38 ns	-0.36 ns	**-0.79** *	0.28 ns				
$MWLR_2$	-0.16 ns	0.54 ns	0.23 ns	0.28 ns	0.04 ns	0.11 ns	-0.36 ns			
$MWLR_3$	**0.82** *	**0.83** *	**-0.83** *	**-0.81** *	-0.44 ns	**0.83** *	0.14 ns	0.21 ns		
MWLR4	**-0.78** *	-0.22 ns	**0.88** **	**0.83** *	0.64 ns	-0.66 ns	-0.57 ns	0.12 ns	-0.67 ns	
CD	**-0.85** *	-0.69 ns	**0.78** *	**0.75** *	0.67 ns	**-0.90** **	-0.30 ns	-0.20 ns	**-0.94** **	0.62 ns

[3] Bold indicates significant correlation. * indicates $P \leq 0.05$. ** indicates $P < 0.01$. *** indicates $P < 0.001$.
ns indicates not significant. *TGA* Thermogravimetric analysis, *DTA* Differential thermal analysis, *TPs* Total phenolics, *CTs* Condensed tannins, *Oil* total oil yield, *RSIT* Relative spontaneous ignition temperature (°C), *MWLR* Maximum weight loss rate (% °C) at peak 1-4, *CD* combustion duration (min). The strong negative correlation between total oil yield and total phenolics, and between 1,8-cineole and total phenolics reflects the intended selection of samples which differed in these traits, whereas a correlation in random samples may differ.

As we had found that water limitation increased concentrations of 1,8-cineole, α-pinene and the total oil yield of juvenile *E. viminalis* leaves (Fig. 2d-f), the concentrations of these traits (extracted from fresh leaf samples) and TPs and CTs (from freeze-dried and ground samples) were correlated against ignition temperature, combustion duration and the combustion rate of each gaseous and solid phase peak (Table 3). Concentrations of 1,8-cineole and total oil yield were negatively correlated with TP concentrations in the assayed samples (Table 3). As such, it was difficult to distinguish whether it was the oil/cineole or total phenolic content of samples influencing the combustion duration, and the combustion rates of peak 3 ($MWLR_3$) and peak 4 ($MWLR_4$) based on these data alone (Table 3). However, high 1,8-cineole and total oil content correlated with lower spontaneous ignition temperatures, while phenolic content of samples did not (Table 3). This result suggests that variation in the spontaneous ignition temperature of samples was due to sample oil content, and mainly reflects variation in 1,8-cineole content as it was the major oil component.

In this experiment, soil water deficit caused little plant water stress, yet the total oil yield increased by 4 mg g^{-1}DW (cineole equivalents). According to a linear regression (Fig. 6), a 4 mg g^{-1}DW total oil increase would reduce the temperature of spontaneous sample ignition by 0.8°C (0.5%). To place this result into context using the same linear regression (Fig. 6) and locality level total oil values (Table 3), average *E. viminalis* leaf samples from KI (wet environment; high total oil yield) would ignite at 1.5°C lower than SH leaves (dry environment; low oil yield), nearly twice the variation that was due to treatment impacts. Even though these samples were purposely selected to maximise oil concentration variation among samples, the reduction in ignition temperature due to a three-fold increase in oil concentration was just 13°C (Fig. 6). Therefore, while water deficit leading to increased oil content could increase leaf ignitability (Fig. 6), greater variation of ignitability was found between localities due to genetic-based variation of constitutive oil concentrations. Furthermore, while we have purposely removed the effect of leaf water content and

leaf morphology from this flammability work, changes to both these traits during water deficit would play a major role in affecting the flammability of juvenile *E. viminalis* during drought.

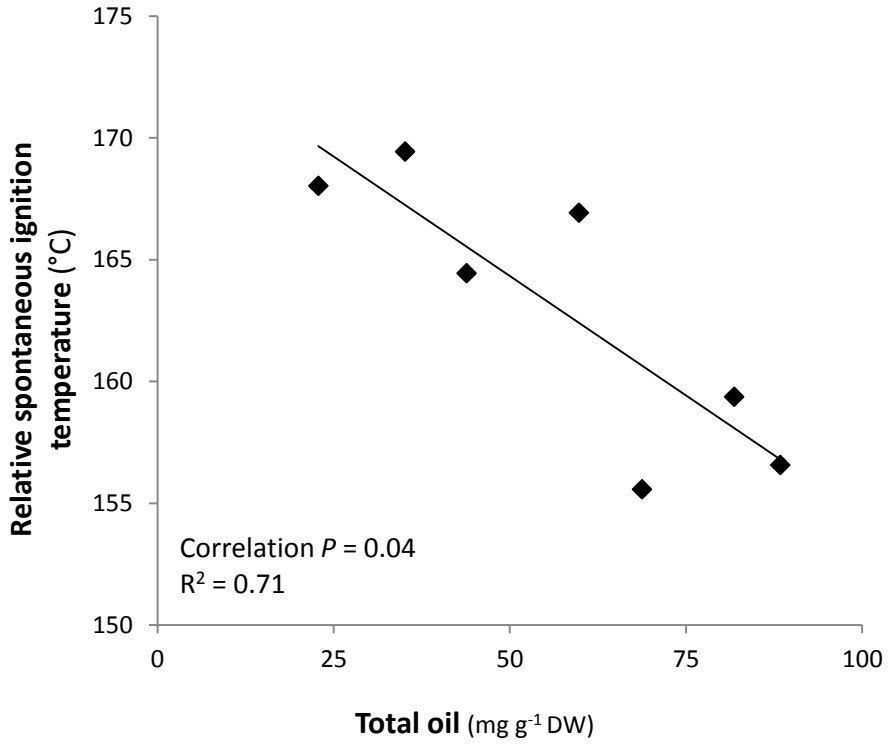

Figure 6. Association between the total oil concentration and the relative spontaneous ignition temperature (RSIT) of freeze-dried and ground juvenile *Eucalyptus viminalis* leaves. RSIT data taken from simultaneous thermogravimetric (TGA) and differential thermal analysis (DTA). Oil extracted from fresh leaves and expressed as $mg.g^{-1}$DW cineole equivalents, TGA-DTA data attained using the same samples, however, leaves were freeze-dried and ground for analysis. Fitted line is a linear regression.

4.5 DISCUSSION

4.5.1 The effect of water limitation on plant traits

The level of water deficit applied (50%) caused little water stress in these juvenile *E. viminalis*, regardless of provenance. We expected Ψ_{leaf} to become more negative and for foliar ABA levels to increase during soil water deficit (Zhang & Davies 1991). While Ψ_{leaf} did become more negative, mean foliar ABA level and plant growth were not affected by the treatments. Despite the moderate nature of the imposed water deficit and the lack of effect on above-ground biomass and foliar ABA levels, the impact of water deficit on leaf chemical traits was substantial. Water deficit increased the total oil yield, increased concentrations of 1,8-cineole and α-pinene (the two major total oil components) and decreased leaf C:N, yet had no significant impact on phenols (total phenolics, condensed tannins, chlorogenic acid, macrocarpals A and G, group of late-eluting FPCs).

The water deficit treatment influenced the concentrations of some terpenes yet not the concentrations of others. The biosynthesis of 1,8-cineole and α-pinene share a common pathway (Group A compounds) compared to the other terpenes we quantified (Group F compounds; Keszei *et al.* 2010). As such, water deficit leading to minimal plant stress appears to have up-regulated an aspect of the biosynthetic pathway common to these Group A terpenes, yet not the pathway leading to synthesis of Group F compounds (globulol and aromadendrene; Keszei *et al.* 2010). Theoretically, severe water deficit should reduce the availability of resources such as carbon, nutrients and water to plants (Chaves *et al.* 2003; Bréda *et al.* 2006), and so the increased 1,8-cineole and α-pinene concentrations during this water deficit treatment is good evidence of unimpeded photosynthesis. Increasing concentrations of phytochemicals during mild water deficit is predicted to occur when water deficit inhibits growth more than photosynthesis (Herms & Mattson 1992). Yet here some terpene concentrations increased while concentrations of other PSMs remained

stable, and growth continued unabated which does not fully support theoretical predictions (Herms & Mattson 1992). Given the important links between terpene concentration and both flammability (Ormeño *et al.* 2009) and herbivory (Lawler *et al.* 1999b), further testing is required to elucidate how more severe water deficit in a natural setting would influence terpene concentrations in juvenile and adult *E. viminalis*, and the potential ecological impacts (Ormeño *et al.* 2009; Bowman *et al.* 2012).

Evidence of the highly varied nature of terpene responses to water deficit can be found throughout the literature. For example, *E. viminalis* total oil yield was reported to be unresponsive to water deficit in Chapter 2, as was the total oil yield of other *Eucalyptus* species including *E. polybractea* (King *et al.* 2004) and *E. camaldulensis* (Leicach *et al.* 2010). In contrast, the total oil yield of *E. camaldulensis* has been shown to change due to water deficit (Doran & Bell 1994), while the 1,8-cineole concentration also increased (Leicach *et al.* 2010). More broadly, water deficit induced terpene accumulation has been reported for a range of taxa including Lamiaceae (Sharafzadeh & Zare 2011), *Pinus* (Llusià & Peñuelas 1998; Turtola *et al.* 2003; Blanch *et al.* 2009), *Quercus* (Blanch *et al.* 2009), *Picea* (Turtola *et al.* 2003), *Pistacia* and *Cistus* spp. (Llusià & Peñuelas 1998), yet increasing terpene concentrations during water deficit are certainly not a universal response (King *et al.* 2004; Leicach *et al.* 2010; Yadav *et al.* 2014). Overall, the diversity of these responses to water deficit among species, PSM classes and individual compounds indicate the complexity of interactions at work, and could also be the effect of non-standardised experimental water deficit among studies.

4.5.2 Changes to plant traits over the duration of sampling

Morphological and chemical traits changed quantitatively over the 56 d sampling period due to normal plant development. Concentrations of TPs, CTs,

chlorogenic acid and 1,8-cineole increased over the experimental duration, while leaf nitrogen content decreased. Increased PSM concentrations suggest that these water limited eucalypts were able to adequately photosynthesise or mobilise stored carbon to maintain plant function (McDowell *et al.* 2008). Developmental changes to PSM concentrations have been documented in a number of species (Elger *et al.* 2009; Badenes-Perez *et al.* 2014) including studies using *Eucalyptus* spp. (Goodger *et al.* 2006; Loney *et al.* 2006b; Goodger *et al.* 2007; Goodger *et al.* 2013). These studies show that *Eucalyptus* terpene concentrations often increase with plant age (Loney *et al.* 2006b; McArthur *et al.* 2010; Goodger *et al.* 2013) as 1,8-cineole concentrations did in this study (Fig. 3c). TP concentrations have been reported to increase over time in *E. nitens* (McArthur *et al.* 2010), yet decrease in *E. froggattii* (Goodger *et al.* 2013). Given that PSM concentrations do change in eucalypts throughout plant development, a more severe level of water deficit which impacts plant morphological development (Chaves *et al.* 2003) and closes stomata (Brodribb *et al.* 2014) is required to test the effect of water deficit on PSM traits throughout ontogenetic changes.

4.5.3 The impact of recovery from soil water deficit on plant traits

Plant recovery from water deficit occurred similarly regardless of the water deficit duration that preceded it. Twelve days of watering to field capacity was sufficient to effectively reverse all changes to leaf traits induced by this level of water deficit, while not inducing any new changes. PSM concentrations in recovered plants were either significantly lower than concentrations in water limited plants (α-pinene), or the concentrations decreased enough to no longer be different from controls (1,8-cineole, total oil yield). Given these recovery effects, terpene levels appear highly plastic over short timescales and may change readily with plant water status. Previously, recovery from water deficit has been described using physiological traits (Blackman *et al.* 2009; Brodribb & Cochard 2009), phytohormone (Brodribb & McAdam 2013) and osmolyte concentrations (Warren *et*

al. 2011), however, few studies have previously quantified changes to non-stress related chemical compounds in regard to recovery (Marchese *et al.* 2010). Here, we show that re-watering after mild water deficit can reverse induced PSM responses within a relatively short 12 d period, yet the ability of PSM concentrations in juvenile *E. viminalis* to be recovered from severe water deficit is unknown.

4.5.4 Impacts of induced terpene accumulation on flammability

Water limitation increased the total oil yield by 7% even though water stress was negligible. Foliar total oil yield was negatively correlated with the temperature at which leaf material spontaneous ignited. While the flammability of plant material has been investigated previously (Alessio *et al.* 2008a; Alessio *et al.* 2008b; Ormeño *et al.* 2009; Leroy *et al.* 2010; Elder *et al.* 2011; Ntoufas *et al.* 2013), few have linked flammability with chemical traits (Alessio *et al.* 2008a; Alessio *et al.* 2008b; Ormeño *et al.* 2009). A number of studies have assayed the flammability of whole leaves (Alessio *et al.* 2008a; Hachmi *et al.* 2011). To eliminate the effects of leaf morphology (Liodakis *et al.* 2008; Liodakis *et al.* 2011) and leaf water content (Alessio *et al.* 2008b) we used standardised samples (freeze-dried and ground) so that only chemical traits differed among samples (Liodakis *et al.* 2008). Using this technique, we provide the first study to use simultaneous TGA/DTA on *Eucalyptus* plant material and correlate results with PSM concentrations in those same samples.

The results we present link high oil yield with increased flammability (ignitability) as previously described in *Pinus* and *Cistus* leaf litter (Ormeño *et al.* 2009). Therefore, not only does plant material become more flammable due to decreased leaf water content during water deficit (Alessio *et al.* 2008a), these juvenile eucalypts may become more flammable based solely on leaf chemical properties. However, the relationship between water deficit, foliar oil yield and flammability needs to be tested using adult *E. viminalis.* Given that wildfires are

generally associated with dry environmental conditions (Bowman *et al.* 2009), the ecological impact of increasingly flammable adult *E. viminalis* could be severe and widespread. The link between specific foliar chemicals and flammability traits clearly requires further study across many species, and overall impacts of PSMs need to be placed into perspective with the effects of other factors in a natural setting such as leaf moisture content.

4.5.5 Conclusion

Using diverse *E. viminalis* germplasm we showed that many plant traits changed quantitatively during plant development, that limited water availability increased terpene concentrations even though water stress was not evident, and that re-watering after water deficit reversed water deficit impacts. This occurred regardless of *E. viminalis* provenance or the duration of the water deficit treatment. However, the water deficit induced chemical changes were of a smaller scale than the quantitative changes to PSM concentrations between sampling times due to plant development. Our initial hypothesis that the duration of water deficit would influence the level of quantitative change to plant traits proved false, likely due to the limited stress induced by the treatment. These responses may differ from those of highly stressed juvenile *E. viminalis*, or to the responses of *E. viminalis* (juvenile and mature) growing in specific environmental conditions at each locality.

Chapter 5

Limits to trait stability in potted juvenile *Eucalyptus* across decreasing levels of soil water availability

This chapter is submitted as:

McKiernan AB, Brodribb TJ, Potts BM, Davies NW, Rodemann T, McAdam SAM, Hovenden MJ, O'Reilly-Wapstra JM. Limits to trait stability in potted juvenile *Eucalyptus* across decreasing levels of soil water availability. *Functional Plant Biology*

5.1 Abstract

Drought is predicted to increase in many regions, and soil water deficit can have severe impacts on plants. Responses to water deficit observed in plant secondary metabolite (PSM) concentrations vary between compounds and also studies, with inconsistent reports of changes to PSM concentrations even within a single species. Juvenile *Eucalyptus globulus* were grown for eight weeks at one of eleven levels of water availability. Physiological, morphological and chemical traits were quantified to assess responses to each level of water deficit. Reducing water availability to 60% did not affect any trait. Supplying 50% or less water decreased leaf water content, 40% or less decreased Ψ_{leaf}, 30% or less increased foliar ABA levels and 20% or less reduced biomass. No water for 8 weeks (0% water) resulted in dead plants, decreased total oil yield and increased leaf C:N, whereas concentrations of phenolics and individual terpenes were not affected by any level of water deficit or plant death. Limits to juvenile *E. globulus* drought tolerance were identified. Plasticity of traits varied, yet concentrations of most PSMs remained stable regardless of water deficit level, even after plant death. Although PSM concentrations remained stable, interactions with other traits which do change during water deficit (e.g. foliar N) may still alter ecological interactions involving PSMs in juvenile *E. globulus*.

5.2 Introduction

Plant secondary metabolites (PSMs) are compounds synthesised by plants and stored in plant organs (Fahn 1979; Kutchan 2005) that can have diverse ecological roles outside of the plant (Wiggins *et al.* 2006a; Ormeño *et al.* 2009; Youngentob *et al.* 2011; Chomel *et al.* 2014). The synthesis of PSMs occurs via multiple biosynthetic pathways (Moore *et al.* 2004; Ashour *et al.* 2010; Petersen *et al.* 2010), yet linking carbon- and nitrogen-based PSM biosynthesis is a requirement for available resources (e.g. carbon, nitrogen, oxygen, hydrogen). For example, leaves of many plants accumulate essential oil, which can consist of a vast array of individual compounds such as terpenes (Keszei *et al.* 2008; Sardans *et al.* 2010) containing much carbon (see section 1.2.1). Formylated phloroglucinol compounds (FPCs) are a group of important phenolic PSMs, and common FPCs include the sideroxylonals (based on dimeric acylphloroglucinols) and macrocarpals (acylphloroglucinols which form adducts with terpenes) (Moore *et al.* 2004). Together with other phenols including condensed tannins of flavonoid origin (Petersen *et al.* 2010), the biosynthesis of both nitrogen-containing (e.g. alkaloids and cyanogenic glycosides) and non-nitrogen containing PSMs (terpenoids and phenolics) is potentially a major drain on plant resources. As such, regardless of differences in the biosynthetic pathways and structure of these PSMs, biosynthesis requires a sufficient supply of resources ultimately obtained from soil water, soil nutrients and atmospheric carbon dioxide (CO_2).

While only some PSMs contain nitrogen (e.g. alkaloids, cyanogenic glycosides), carbon, oxygen and hydrogen are essential components of all PSMs (Wink 2010). A reduction in the availability of these essential resources impacts plant growth (Mokotedi 2010; Pinkard *et al.* 2011), and should theoretically also impact PSM synthesis and PSM accumulation (Herms & Mattson 1992). Soil water content decreases during drought, and as root water uptake and within-plant water transport declines, the uptake and transport of nutrients such as nitrogen also declines (McDowell *et al.* 2008). When root water uptake is limited, plants reduce the loss of

within-plant water by closing stomata (McAdam & Brodribb 2014). Stomatal aperture is regulated by plants using the phytohormone abscisic acid (ABA), the levels of which increase during drought and reduce guard cell turgor, closing stomata (Tardieu & Simonneau 1998; Bauer *et al.* 2013; McAdam & Brodribb 2014). In controlling stomatal aperture, plants can balance water loss while stomata are open against CO_2 acquisition (Maximov 1929; McDowell *et al.* 2008; Brodribb & Cochard 2009). However, prolonged water deficit can bring about a negative within-plant carbon balance, when carbon assimilation is inhibited due to stomatal closure and within-plant carbon stores are depleted (McDowell *et al.* 2008). Therefore, even though plants can minimise stomatal water loss, decreasing levels of available water in the soil should fundamentally impact the ability of plants to access resources and, as such, a certain level of water deficit could lead to decreased PSM biosynthesis (Herms & Mattson 1992).

The literature contains contradictory reports of water deficit impacts on PSM concentrations, with opposing impacts found across different species (Pizarro & Bisigato 2010), but also within species such as *Eucalyptus camaldulensis* (Miles *et al.* 1982; Doran & Bell 1994; Leicach *et al.* 2010). For example, the concentration of 1,8-cineole in *E. camaldulensis* can either increase (Leicach *et al.* 2010) or decrease (Doran & Bell 1994) due to water deficit. However, previous work has also shown that water deficit can have minimal impacts on PSM concentrations in juvenile eucalypts (Chapter 2). Of particular interest are reports of increased PSM concentrations due to water deficit, as this indicates that even though resources are limited, PSM accumulation continued while other traits were negatively impacted (Leicach *et al.* 2010; Azhar *et al.* 2011; Yadav *et al.* 2014). Herms and Mattson (1992) suggested that any resource limitation that inhibits growth more than photosynthesis would increase the resources available for PSM synthesis. For example, growth may be inhibited by moderate water deficit yet photosynthesis can continue which results in a carbon surplus (Herms & Mattson 1992), that may be used to increase PSM concentrations (Leicach *et al.* 2010; Azhar *et al.* 2011; Yadav

et al. 2014). As such, variation in PSM responses to water deficit among studies may arise from differences between species tolerance strategies, but also variation in the levels of experimentally applied water deficit.

In order to thoroughly investigate the impact of water deficit on PSM concentrations, and assess specific plant physiological and morphological responses, a single species of natural and commercial value was chosen. *Eucalyptus globulus* is grown commercially worldwide (Potts *et al.* 2004), and is also widespread in natural stands across south-eastern Australia (Dutkowski & Potts 2012). *Eucalyptus globulus* foliage contains many groups of PSMs such as phenolics (including tannins and FPCs) and essential oil (containing multiple terpenes) which have previously been quantified in a number of observational and experimental studies (e.g. Eyles *et al.* 2003b; Close *et al.* 2004; O'Reilly-Wapstra *et al.* 2004; Rapley *et al.* 2007; Smith *et al.* 2007; Freeman *et al.* 2008; O'Reilly-Wapstra *et al.* 2010). However, predicted increases in the frequency and severity of drought periods (Dai 2013) might influence PSM accumulation, and also affect PSM mediated interactions such as herbivory (Wiggins *et al.* 2006a) and plant flammability (Ormeño *et al.* 2009). While some studies have investigated the influence of water deficit on *E. globulus* osmolytes (Merchant *et al.* 2006; Shvaleva *et al.* 2006), few studies have investigated *E. globulus* PSM concentrations in relation to water deficit. The only previous work described how limited water availability decreased total phenolic concentrations in leaves of juvenile *E. globulus*, yet did not affect concentrations of most other PSMs (Chapter 2). To test whether most PSM concentrations remained stable in this earlier experiment because the water deficit treatment was not severe, this experiment uses multiple levels of water deficit to ascertain the water deficit level at which physiological, morphological and chemical traits are impacted.

Here, we used juvenile *E. globulus* of five provenances from across the species range in a glasshouse-based trial to investigate the impact of ten different water deficit levels on plant physiology, growth and leaf chemical traits. We used

juveniles of multiple provenances rather than limited genotypes in order to better test the species level response. We were specifically interested in determining the levels of soil water deficit at which different traits were impacted. We reasoned that a specific level of water deficit (i.e. one of the treatments) would cause *E. globulus* stomatal adjustment to reduce water loss from leaves and that carbon assimilation would subsequently be inhibited (McDowell *et al.* 2008), leading to reduced growth and/or PSM accumulation compared to control plants. The specific aim of this study was to determine the level of water deficit required to influence growth and PSM concentrations of juvenile *Eucalyptus globulus*.

5.3 Materials and Methods

5.3.1 Plant material

Eucalyptus globulus seed of five provenances were collected from multiple trees ($n \geq 3$) at each locality (one genetic provenance at each geographic locality). Localities are Southern Tasmania, Queens Domain, St Helens and King Island (described in section 3.3.1) with the addition of Jeeralang North from south-eastern Victoria (for location details see Dutkowski & Potts 1999). These localities were selected as they encompass a large range of *E. globulus* genetic-based drought tolerance (mature trees; Dutkowski & Potts 2012) and diverse foliar chemical profiles (O'Reilly-Wapstra *et al.* 2004; Wallis *et al.* 2011). Open-pollinated seed obtained from a single mother tree was pooled and termed a 'family'. Mother trees were chosen from across each location, and were at least 100 m apart to limit sampling from highly related individuals and to maximise genetic variation in the selected trees of each provenance.

Eucalyptus globulus seed from all mother trees were germinated and grown for 1 month. Uniform size (cotyledons and 1 leaf pair) seedlings (n=110 total) from each locality were selected and transplanted into individual plastic pots (base 38 x 38 mm, top 50 x 50 mm, height 118 mm). Potting mix contained eight parts composted fine pine bark: three parts coarse river sand, and N:P:K [19: 2.6: 10] at 1 g/L potting

mix. The pH was adjusted to approximately 6.0 with the addition of dolomite lime at 3 kg/m^{-3}. Seedlings were grown for 12 weeks, then re-potted into larger pots (base 115 mm diameter, top 138 mm diameter, height 250 mm) containing exactly 900 g of potting mix (430 g dry mass) with the addition of extra fertilizer (5 g Osmocote® 3-4 month [N14:P6.1 :K11.6] per pot) and wetting agent (Everris Hydraflo®) added at 1.35 L/m^{-3} potting mix. All 110 pots contained equal bulk density of potting mix and a single eucalypt. Potted plants were arranged in a randomised block design in a naturally lit glasshouse (midday light c.1350 µmol m^{-2} s^{-1}; midday temperature c. 26°C), where plants of each locality (randomly selected with respect to family) were randomly but evenly divided between treatments and replicate blocks. Plants of each locality and treatment were completely randomised within each replicate, watered daily, and grown for a further six weeks before experimental treatments were applied.

5.3.2 Experimental treatments

Treatments began in February 2013 (summer) when eucalypts were 6 months old, and followed methods outlined by Mitchell *et al.* (2013). One of eleven different watering regimes (treatments) was allocated to plants (Table 1). Treatments were control (fully watered to field capacity daily [100% water treatment]) and 10 levels of water deficit, ranging in 10% increments from 90-0% replacement of mean evapotranspired water from control plants. Field capacity (g) was determined after saturating each control pot with water, then allowing excess water to drain until dripping ceased (~30 min). First, water was withheld from all plants in water deficit treatments (controls continued to be watered) for 3 d to reduce pot water content and cause initial plant stress. After 3 d, most of these un-watered plants showed signs of turgor loss as indicated by wilting. Evapotranspiration was monitored daily (gravimetrically) on six control plants during this initial 3 d period (controls were watered daily to field capacity), and a percentage of the rolling daily mean from 3 d was calculated. The pre-determined percentage of the mean evapotranspiration from controls (90-10%) was then multiplied by two (as plants were not watered daily) and

applied to each pot in the respective treatment every other day, creating a pulsed but continuous level of water availability (water provided = mean control evapotranspiration × treatment percentage [between 0.9 and 0.1] × 2 days; Table 1). The control pots used for determination of evapotranspiration were selected randomly and were re-selected weekly. Evapotranspiration of controls continued to be recorded daily throughout the 8 weeks, and continuously updated percentages of this mean evapotranspiration were applied to plants in each treatment. Due to the hydrophobic nature of dry potting mix, clear plastic bags were placed over the base of all pots receiving limited water (90-0%) to eliminate drainage of applied water without inhibiting evaporation from the soil surface. Plants at no stage were waterlogged. Bags were not used on control pots as excess water was required to drain for pot water content to reach field capacity. Treatments were maintained for 8 weeks before plants were harvested.

Table 1. Mean (±SD) amount of water applied to juvenile *Eucalyptus globulus* in each of the ten water deficit treatments (90 - 0%) every 2 days over 8 weeks[1]

Treatment (% of control)	Number of plants	Water applied (mL)
100 (Control)	10	*268 (86)*
90	10	241 (77)
80	10	215 (69)
70	10	188 (60)
60	10	161 (52)
50	10	134 (43)
40	10	107 (34)
30	10	81 (26)
20	10	54 (17)
10	10	27 (9)
0	10	0 (0)

[1]Mean water applied to controls (italics) represents the mean amount of water required to return pots to field capacity after water was lost through evapotranspiration. Controls were watered daily and daily evapotranspiration recorded. Mean control evapotranspiration of the previous 3 d was used to calculate water applied to deficit treatments every second day, calculated as the percentage of the control mean multiplied by 2 d. Control SD value indicates variation in evapotranspiration due to local weather variability, and as all other treatments were calculated from control water loss, there was also SD in water applied to treatments. Plants in the 0% water treatment received no water for 8 weeks.

5.3.3 Sample collection

Sampling occurred over 3 d, with sampling not undertaken on the second day due to the plants in the 90-10% treatments being watered that day. Therefore, water limited plants were not watered and control plants were watered to field capacity the morning prior to harvest. Each sampling day (n=2), half of the juvenile plants of all treatments (1 plant per provenance) were placed in full sunlight for >30 min (total = 55 plants per day). A subset of plants (n=50 total) were randomly selected *a priori* for measurement of leaf water potential (Ψ_{leaf}) in order to quantify the impact of water deficit on plant stress (Brodribb & McAdam 2011). A single fully-formed juvenile leaf (25 plants per sampling day) was taken in full midday sunlight (1500-2000 µmol m^{-2} s^{-1}) and Ψ_{leaf} quantified using a Scholander pressure chamber (PMS, Albany, OR, USA). All plants were assessed for foliar abscisic acid level (ABA; a phytohormone which signals stomatal closure during water limitation) as a proxy for stomatal closure (McAdam & Brodribb 2014). On each of the two sampling days, the 5 plants in each treatment (n=11 treatments) were randomly divided into two groups (two or three random plants with respect to locality), a fully-expanded juvenile leaf was taken from a uniform position on each plant, leaves from the two-three plants pooled (~3 g leaf total, two pooled samples per treatment) and immediately immersed in liquid nitrogen (44 pooled samples total), then stored at -70°C until ABA quantification. Sampling over two days provided four pooled ABA samples per treatment.

All plants were then harvested individually using the following method. The stem was cut and total fresh above-ground biomass was determined. We were interested in how the treatments affected leaf traits, and so harvested only the uppermost eight fully-formed leaf pairs of each plant, disregarding leaves which were not fully-expanded or were damaged. This sampling method was employed because growth is reduced as water availability decreases (Chapter 2), and healthy *E. globulus* often abscise lower leaves during normal development, so sampling from

consistent nodes across treatments was not possible. Therefore, harvested leaves of control plants mostly expanded during the treatments, while harvested leaves of 10 - 0% water plants expanded prior to the treatments, with the proportion of leaves in a sample which developed prior to treatment application decreasing as water availability in a treatment increased. The eight leaf pairs were stripped from the stem, pooled and mixed. A random sub-sample was frozen (-12°C) for essential oil analysis, then remaining leaves were weighed, frozen, freeze dried, re-weighed, scanned using near infrared spectroscopy (NIRS – see below), ground in a Cyclotec™ 1093 cyclone mill (Foss, Hillerød, DK) and passed through a 1 mm sieve for analysis of primary chemistry (Thermo Finnigan EA 1112 Series Flash Elemental Analyser, Italy), total phenolics, condensed tannins, chlorogenic acid and FPCs. Sample mass prior to and post lyophilisation was used for quantification of fresh leaf water content (100-[dry mass/fresh mass ×100]), expressed as percentage fresh leaf weight (%FW). The fresh stem and un-harvested leaves were weighed and oven dried at 40°C until constant mass. Total dry above-ground biomass was determined using dry stem and leaf mass, fresh total above-ground biomass, and the calculated dry mass of harvested leaves.

5.3.4 Abscisic acid (ABA)

Extraction of foliar ABA followed the method described by McAdam and Brodribb (2014) except that ~0.2 g of pooled leaves were used per sample. Endogenous ABA was quantified using a Waters ultra-performance liquid chromatograph coupled to a Xevo triple quadrupole mass spectrometer (UPLC-MS), with results expressed as ng g^{-1} FW.

5.3.5 Quantification of secondary chemistry

Extraction, analysis, quantification and identification of essential oil components (1,8-cineole, α-pinene, globulol, aromadendrene and limonene), and quantification of the total oil yield followed the method outlined by McKiernan *et al.* (2012). Concentrations of 1,8-cineole and α-pinene were expressed as mg g^{-1} DW

(dry weight) using standards, while total oil yield and other oil components were expressed as mg g^{-1} DW cineole equivalents. Phenols were extracted, then total phenolics (TPs) and condensed tannins (CTs) were quantified in duplicate as described in Chapter 2, with the exception that a VIS-7200A UV-visible spectrophotometer (Techcomp Limited, Hong Kong) was used. Total phenolic results were expressed as mg g^{-1} DW gallic acid equivalents, CT results were expressed as mg g^{-1} DW sorghum tannin equivalents. We assayed two specific FPCs (macrocarpals A and G) by High Performance Liquid Chromatography (HPLC) following methods of Wallis and Foley (2005). A group of relatively late-eluting FPCs (eluting just before and after macrocarpal G) were also quantified. Macrocarpal A was expressed as mg g^{-1} DW, while macrocarpal G and the group of late-eluting FPCs was expressed as mg g^{-1} DW macrocarpal A equivalents, using standards described by Eyles *et al*. (2003a). Chlorogenic acid was extracted and quantified as described in Chapter 3 (section 3.3.7).

5.3.6 Near infrared spectroscopy (NIRS)

Near Infrared Spectroscopy (NIRS) and wet chemical values (from a subset of samples) were used to create predictive models, and then to predict chemical values of each trait in the remaining samples as described previously (section 3.3.8). Samples from plants that received no water (0% water treatment) were desiccated and brittle, making them unsuitable for whole leaf NIRS analysis. Instead, quantitative chemical analysis of each trait was carried out on all samples from the 0% water treatment (n=10) concurrently and randomly with the samples used for model calibration and validation (Chapter 3; Table 2). The 0% water samples were not included in NIRS modelling, but data from this treatment was included for statistical analysis.

5.3.7 Statistical analysis

Although primarily interested in the treatment effects on plant traits, we included locality in the analysis to test for trait variation among provenances. Testing of fixed effects (treatment, locality) was undertaken using various general linear models (PROC GLM of SAS) depending upon the data set. As Ψ_{leaf} data were from limited plants (n = 50), and ABA data was pooled samples rather than from a single plant (4 pooled samples per treatment = 44 pooled ABA samples total), only treatment (n=11 levels of water availability) was fitted in the model for these traits. For all other traits, treatment, locality (n= 5) and the interaction were fitted. Residuals for all variables were checked for assumptions of normality and heterogeneity of variances, and transformations made where necessary. ABA, total oil, α-pinene, 1,8-cineole and limonene data were transformed to their natural logarithm. Due to the number of individual analyses performed (n=17), the false discovery rate was controlled following Benjamini and Hochberg (2000). In order to summarise the multivariate patterns of chemical variation between treatments, all PSM traits were analysed using discriminant analysis (PROC DISCRIM of SAS) with each treatment treated as a separate group.

5.4 Results

Water deficit had strong impacts on juvenile *E. globulus* physiological and morphological traits (Table 2), and the effect was dependant on the level of water deficit. We also found trait variation between localities, yet all localities responded the same to the treatments (i.e. no locality × treatment interaction) regardless of the variation in drought tolerance among mature *E. globulus* from these provenances (Dutkowski & Potts 2012) and constitutive trait variation between localities (Table 2). As such, only results of the treatment effect are discussed, with trait variation between localities presented in Supplemental Table S6.

Table 2. Results of general linear model (GLM) analysis of juvenile *Eucalyptus globulus* trait variation between localities (KI, ST, QD, SH, JN), treatments (11 levels of water availability) and the interaction[2]

	Locality		**Treatment**		**Locality × treatment interaction**	
	$F_{4, 107}$	*P*	$F_{10,107}$	*P*	$F_{40,107}$	*P*
Above-ground biomass	4.7	**	12.8	***	1.4	
Leaf water content	1.8		274.8	***	0.5	
Carbon	25.0	***	1.7		1.4	
Nitrogen	0.4		3.7	***	0.8	
C:N	0.4		4.9	***	0.8	
1,8-Cineole	8.9	***	0.8		0.8	
α-Pinene	5.8	***	1.3		0.8	
Aromadendrene	10.4	***	3.2	**	1.4	
Globulol	11.4	***	2.4	#	1.3	
Limonene	19.2	***	0.6		0.9	
Total oil yield	13.9	***	3.7	***	0.8	
Chlorogenic acid	8.6	***	0.6		0.6	
Condensed tannins	8.9	***	1.2		0.9	
Total Phenolics	0.6		0.3		0.8	
Macrocarpal A	31.5	***	1.4		1.2	
Macrocarpal G	36.1	***	2.2	#	1.4	
Late-eluting FPCs	18.9	***	1.3		1.3	

[2]*KI* King Island. *ST* Southern Tasmania. *QD* Queens Domain. *SH* St Helens, *JN* Jeeralang North

*indicates $P \leq 0.05$. ** indicates $P < 0.01$. *** indicates $P < 0.001$.

[#] indicates $P \leq 0.05$ but not significant after correction for false discovery rate (Benjamini & Hochberg 2000)

5.4.1 Above-ground biomass and leaf water content

Water deficit decreased the biomass of juvenile eucalypts grown in 20-0% water (93-10 g^{-1} DW) compared to the mean biomass of controls (242 g^{-1} DW), and also compared to eucalypts in the 90-30% water treatments (Table 2, Fig. 1a). Plants provided 90-30% water showed no significant reduction in biomass, yet the trend suggests that the impact of water deficit on growth may have begun in *E. globulus* provided 40% water (204 g^{-1} DW; Fig. 1a). The leaf water content of *E. globulus* given 50-0% water (65-7.3% FW) was lower than in controls (71% FW; Fig. 1b). Specifically, while the mean leaf water content of eucalypts in the 50-10% treatments decreased by 5-15%, *E. globulus* in the 0% water treatment died (no water for 8 weeks), and leaf water content decreased by 90% from control levels, to only 7.3% of fresh leaf mass (Fig. 1b).

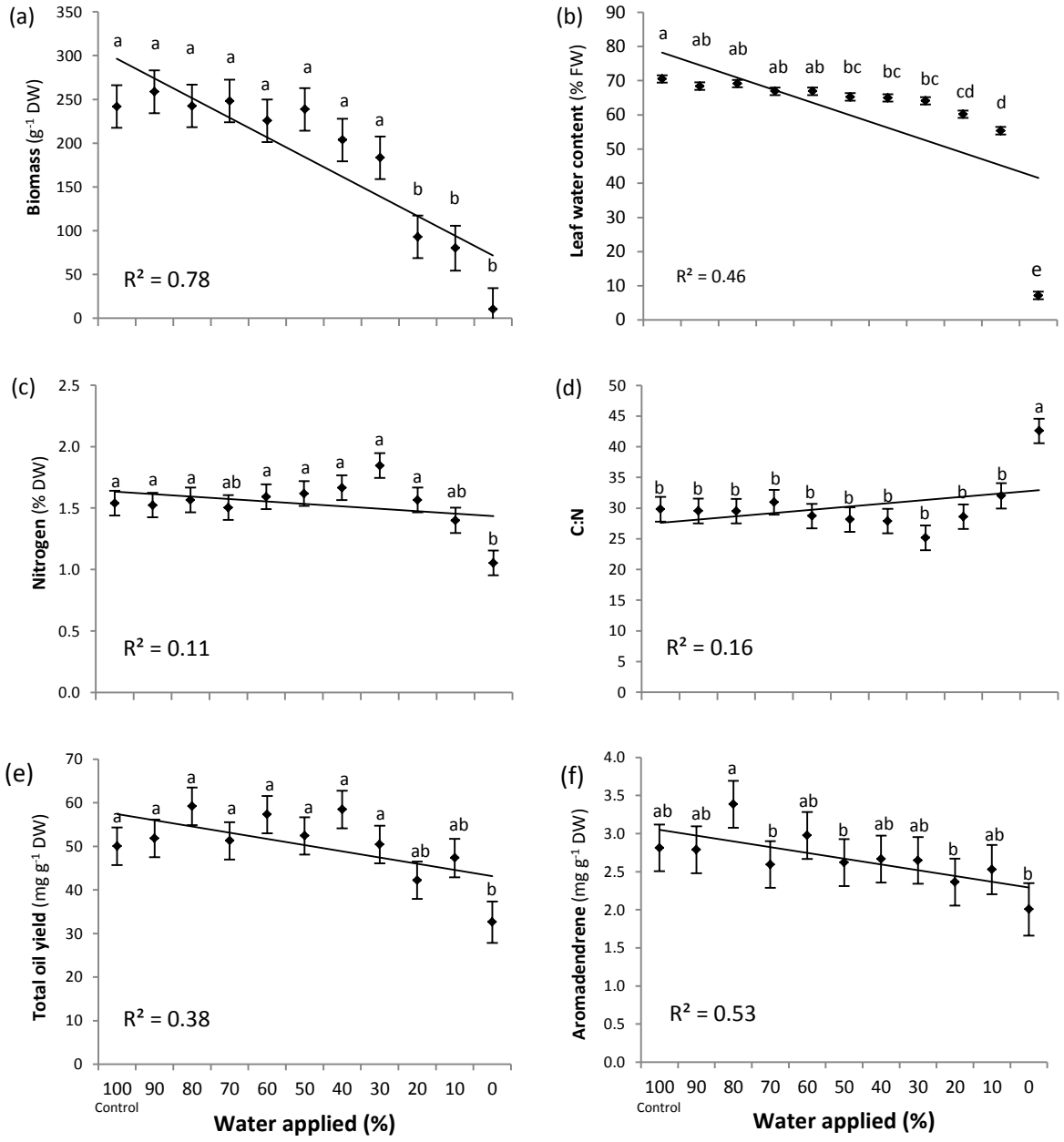

Figure 1. LSmean values of (a) above-ground biomass, (b) leaf water content, (c) nitrogen, (d) carbon to nitrogen ratio, (e) total oil yield and (f) aromadendrene in juvenile *Eucalyptus globulus* leaves between treatments (water applied %). Treatments were based on the amount of water loss from pots/plants via evapotranspiration from controls (100% water). Bars indicate standard error. Different letters within each graph indicate significant difference ($P \leq 0.05$) after Tukey's *post hoc* tests. Note scale variation on y-axis. Linear regression line based on the means of each treatment (shown).

5.4.2 *Leaf water potential* (Ψ_{leaf}) *and abscisic acid* (ABA) *level*

Soil water deficit had a dramatic impact on Ψ_{leaf}, which became significantly ($F_{9,49}$=26.54, P<0.0001) more negative in treatments receiving 30% or less water (Fig. 2a). Juvenile eucalypts receiving just 10% of the water used by controls had mean Ψ_{leaf} of -2.57 MPa. While the mean Ψ_{leaf} of plants which received 40% water (-0.9 MPa) did not differ statistically from controls (-0.3 MPa), the mean Ψ_{leaf} fell below the range of control values (±SE) and appears to be the point after which Ψ_{leaf} became increasingly negative (arrow in Fig. 2a). No Ψ_{leaf} data were collected from 0% water plants as eucalypts in this treatment died. Foliar ABA level also varied between treatments ($F_{9,39}$=4.60; P=0.0007), but the response trend was more gradual than seen in the Ψ_{leaf} responses (Fig. 2b). The mean foliar ABA levels ranged nearly three-fold between treatments, from 319 (ng g^{-1} FW) in controls (100% water) up to 1092 (ng g^{-1} FW) in plants that received only 10% of control water (Fig. 2b). Due to the death of 0% water treatment plants, the low leaf water content (7.3%) and the expression of ABA levels in terms of fresh leaf mass (ng g^{-1} FW), ABA data from eucalypts in the 0% water treatment were excluded from the analysis and from Fig. 2b. However, the mean foliar ABA level of fresh yet desiccated (due to the treatment) leaves from plants in the 0% water treatment was 3102 ng g^{-1} FW.

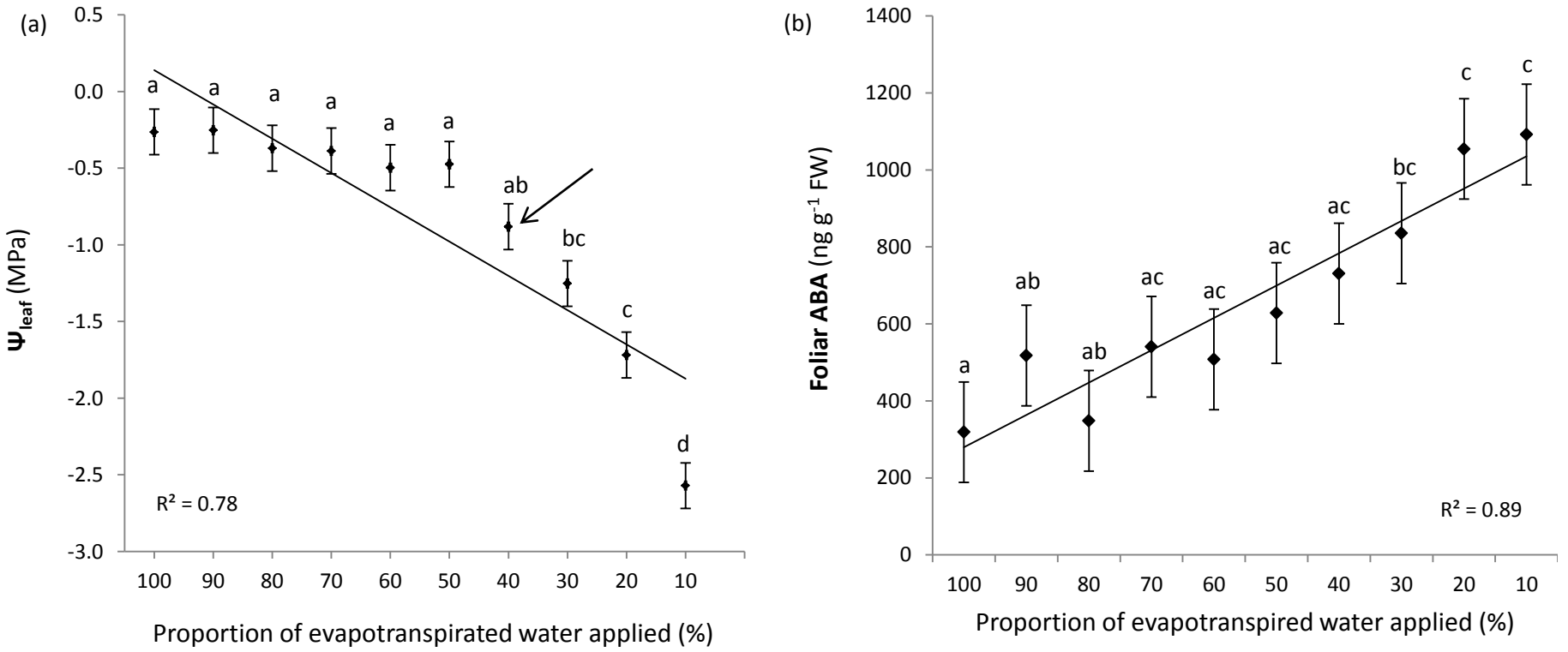

Figure 2. LSmean values for (a) leaf water potential (Ψ_{leaf}) and (b) foliar abscisic acid (ABA) level in juvenile *Eucalyptus globulus* leaves between treatments (% water applied). Treatments were based on the amount of water lost via evaporation and transpiration from controls (100% water). Bars indicate standard error. Different letters within each graph indicate significant difference among treatments ($P \leq 0.05$) after Tukey's *post hoc* tests. Note scale variation on *y*-axis. Trend-line is a linear regression. Arrow on (a) indicates point at which Ψ_{leaf} exponentially becomes more negative.

5.4.3 Primary chemistry

While still affected by the treatments (Table 2), primary chemistry was less responsive to water deficit than Ψ_{leaf} and foliar ABA level. Leaf N content and the C:N were affected only when water was completely withheld (0% treatment) for 8 weeks (Fig. 1c, d). Specifically, 0% water decreased N by 32% (Fig. 1c), and increased the C:N by 30% compared to controls (Fig. 1d), yet no change was detected due to any other treatment (90-10% water) (Fig. 1c). No level of water deficit affected leaf C levels (Table 2).

5.4.4 Terpenoids

Concentrations of the total oil yield and aromadendrene were reduced by the watering treatments, yet concentrations of other terpenes including 1,8-cineole, α-pinene, globulol and limonene remained stable regardless of any level of soil water deficit (Table 2). Water deficit only influenced the total oil yield of leaves from plants in the 0% water treatment, which contained significantly lower total oil than control plants (33 mg g^{-1} DW and 50 mg g^{-1} DW cineole equivalents, respectively) and eucalypts in the 90-30% water treatments (Fig. 1e). The overall trend of total oil concentrations was quite variable in the 100-30% treatments, but total oil appeared to begin decreasing in plants of the 20% water treatment (42 mg g^{-1} DW cineole equivalents). Aromadendrene concentration was found to significantly differ between treatments (Table 2), however, *post-hoc* tests showed that no concentration in water deficit treatments differed to controls, and the differences detected involved different levels of water deficit (Fig. 1f). Specifically, aromadendrene concentrations were higher in *E. globulus* grown under 80% water (3.4 mg g^{-1} DW cineole equivalents) compared to aromadendrene concentrations in leaves of eucalypts in the 70%, 50%, 20% and 0% water treatments (Fig. 1f). Given the pattern of variation between treatments, we concluded that aromadendrene concentrations were influenced by water availability. Perhaps a clearer trend of decreasing aromadendrene concentrations as water availability decreases could be identified with further tests (Fig. 1f).

5.4.5 Phenolics

There was no impact of any level of soil water deficit on the bulk phenolic groups (TPs, CTs and late-eluting FPCs; Table 2). Likewise, concentrations of individual phenols (chlorogenic acid, macrocarpal A and macrocarpal G) were unaffected by any level of soil water deficit, with concentrations remaining stable in leaves of *E. globulus* provided just 10% water, and even in plants which died after not being watered for 8 weeks (Table 2).

5.4.6 Ordination

The multivariate analysis investigated differences between treatments using all PSMs extracted in this study. The resulting multivariate plot showed that water deficit only affected the overall PSM concentrations of juvenile *E. globulus* leaves when the amount of water provided was below 50% of control evapotranspiration (Fig. 3). Providing 90-60% water had no impact on overall foliar PSM concentrations, reflecting the stability of individual PSM concentrations detected when each chemical trait was analysed separately (Table 2, Fig. 1e-f). Severe water limitation (10-0% water) appeared most linked with the x-axis (cv1) which explained 72% of the total PSM variation between treatments, whereas the 50% and 30-10% water treatments separated from controls along both cv1 and cv2, where cv2 explained 9% of the overall variation in PSM concentrations (Fig. 3).

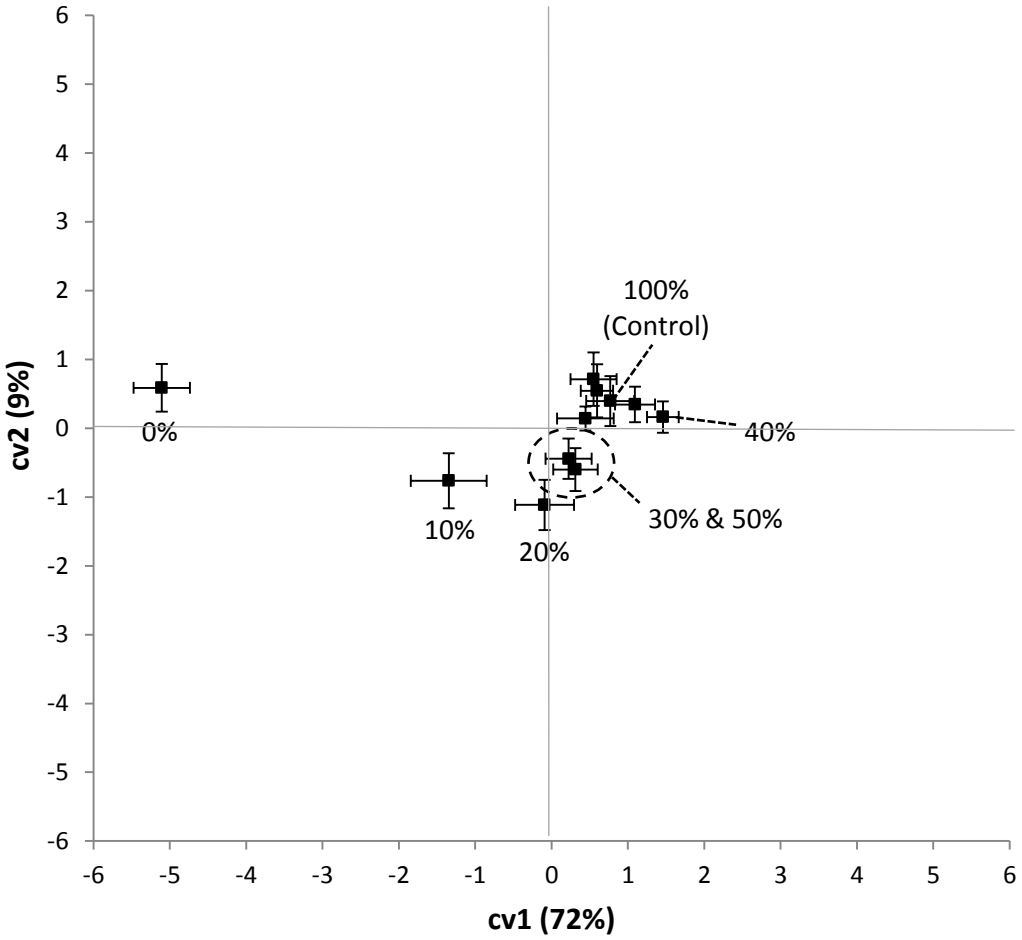

Figure 3. Ordination summarising the overall response of juvenile *Eucalyptus globulus* leaf chemical traits to eleven levels of water availability ranging from 100% (controls) to 0% (no water for eight weeks) in 10% increments. The axes are the first two canonical variates derived from discriminant analysis, summarizing the overall response of macrocarpal G, macrocarpal A, late-eluting FPCs, total phenolic, condensed tannin, chlorogenic acid, 1,8-cineole, α-pinene, limonene, aromadendrene, globulol and total oil yield. Only controls and treatments which varied from controls ($P \leq 0.05$) are labelled. Bars indicate standard error (±SE) along each axis. Percentages along each axis indicate the proportion of variation explained by each canonical coefficient. 1,8-Cineole, α-pinene, limonene and total oil yield data were transformed to their natural logarithm.

5.5 Discussion

The use of multiple levels of soil water deficit enabled us to assess the variation in responsiveness of physiological, morphological and chemical traits in juvenile *E. globulus*. The sequence of responses to increasing water deficit are summarised in Figure 4. We found that reducing water availability to 60% of control evapotranspiration had no impact on any measured plant trait (Fig. 4). Leaf water content decreased in plants provided 50% water, and the multivariate PSM profile changed. Reducing water availability to 40% of the control level significantly decreased mean Ψ_{leaf}. Mean foliar ABA level increased steadily as water decreased, and plants in the 30% water treatment had a mean foliar ABA level significantly higher than controls, indicating the induction of plant mechanisms to close stomata and reduce leaf water loss (McAdam & Brodribb 2014), and thus decrease productivity and growth. Changes to morphological traits were detected only when water availability was at or below 20% of control evapotranspiration. Above-ground biomass significantly decreased when plants were provided 20% water; however, the trend suggests that growth began to decrease even in the 40% water treatment (Fig. 1a). Reducing water availability below 20% had no impact on individual chemical traits until the 0% treatment, which caused plant death, decreased the total oil yield and increased leaf C:N. Unexpectedly, no level of soil water deficit affected concentrations of assayed phenolics (TPs, CTs, chlorogenic acid or FPCs) in these juvenile *E. globulus*.

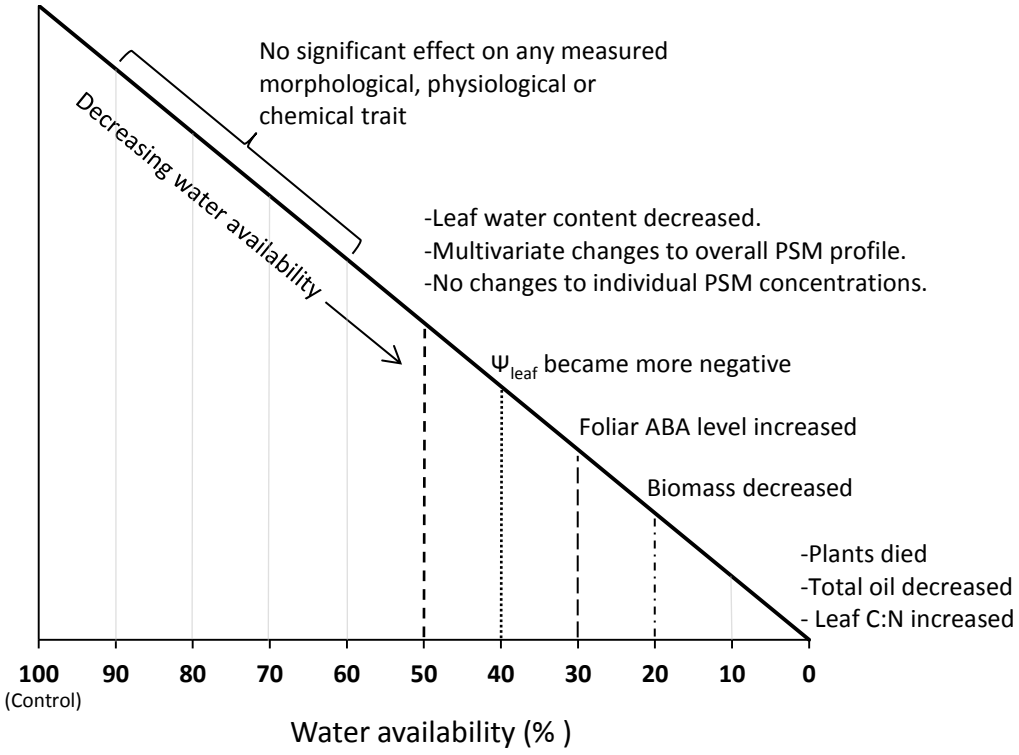

Figure 4. Summary of the changes to juvenile *Eucalyptus globulus* traits with decreasing water availability.

5.5.1 Limits to juvenile Eucalyptus globulus *tolerance of soil water deficit*

Juvenile *E. globulus* were able to tolerate a 50% reduction in water availability without Ψ_{leaf} becoming more negative, and with no increase in foliar ABA levels. Foliar ABA levels were elevated in *E. globulus* grown under 40-0% water, indicating that these treatments induced hormonal regulation of stomatal aperture (Zhang & Davies 1991; Bauer *et al.* 2013; McAdam & Brodribb 2014) until stomata were closed, after which leaf water potential declined with soil water potential (Fig. 2a, b). As such, a 50% reduction from the optimum amount of water these eucalypts could use/lose represents a threshold to effective transpiration control (because after this point the stomata were closed). Below this 50% water threshold, juvenile *E. globulus* water deficit tolerance may rely on other traits such as those which provide resistance to vascular cavitation (Tyree & Sperry 1989; Hacke & Sperry 2001; Cochard *et al.* 2007; Brodribb & Cochard 2009).

Limits to *E. globulus* tolerance of soil water deficit have been documented previously (Costa e Silva *et al.* 2004; Gindaba *et al.* 2004; Mitchell *et al.* 2013; Granda *et al.* 2014; Mitchell *et al.* 2014) and reflect the responses presented here. For example, *E. globulus* radial stem growth ceases when Ψ_{leaf} falls below -1.4 MPa (Mitchell *et al.* 2014), and here we show that above-ground biomass decreased when mean Ψ_{leaf} reached -1.7 MPa in the 20% water treatment. We also know from previous work that *E. globulus* carbon assimilation ceases when Ψ_{leaf} is between -2.5 MPa and -3.0 MPa (Costa e Silva *et al.* 2004; Gindaba *et al.* 2004; Mitchell *et al.* 2013; Mitchell *et al.* 2014). Here, the Ψ_{leaf} of juvenile *E. globulus* in the 10% treatment reached -2.5 MPa and, therefore, we expect that assimilation was very low or had ceased in eucalypts from the 10% water treatment. Given that eucalypts which received very little water (20-10% water of controls) had reduced growth, the reduced C demand (for above-ground growth) may have created a C surplus (Herms & Mattson 1992; Mitchell *et al.* 2014) which could then be allocated to other essential traits not quantified here, such as increased root growth (Jackson *et al.* 2000).

5.5.2 *Water deficit impacts on juvenile* Eucalyptus globulus *leaf chemical traits*

The concentrations of all assayed PSMs remained stable when provided 90-10% the quantity of water used by controls. We expected that in some water deficit treatments, reduced water availability would limit new resource acquisition (Burns *et al.* 2002) and, therefore, alter PSM concentrations. It is likely that juvenile *E. globulus* in the 30-10% water treatments had decreased C assimilation based on the high foliar ABA level which indicates stomatal closure (Fig. 2a), and based on other work investigating hydraulic conductance and carbon assimilation in this species (Costa e Silva *et al.* 2004; Gindaba *et al.* 2004; Mitchell *et al.* 2013; Mitchell *et al.* 2014). Due to the likely reduction in C acquisition of eucalypts in the 30-10% water treatments, it appears from the stability of PSM concentrations that these PSMs were not being used as resources to be re-metabolised for other roles (Wink 2010) during water deficit. As concentrations of these important PSMs remained stable even in severely water stressed juvenile *E. globulus* with limited photosynthetic ability, PSM accumulation in leaves of these *E. globulus* may have plateaued during plant development and before water deficit was applied. As such, we may have detected an effect of water deficit on PSM concentrations if we had applied the treatments when the eucalypts were at an earlier developmental stage (these eucalypts were 6 months old at the time treatments began), as PSM concentrations change rapidly in early eucalypt development (Borzak *et al.* 2015). Due to differences in plant growth between treatments, it was not possible to harvest leaves for chemical analysis from consistent nodes across treatments. In doing so, when we compare PSM concentrations between treatments, we unavoidably include PSM concentration variation between leaves of the different nodes of different ages (Chapter 2). Nevertheless, with respect to this sampling variation, we conclude that after 8 weeks of water deficit (90-10% water) there was no difference in PSM concentrations of the eight most recently fully-expanded leaf pairs of these juvenile *E. globulus*.

Eucalypts in the 0% water treatment received no water for 8 weeks and died during the experiment, leaving desiccated non-functional leaves, and yet

concentrations of most PSMs in leaves of these plants were comparable with concentrations in leaves of well-watered control plants. The only effect of complete water deficit on PSM concentrations was to decrease the total oil yield. As *Eucalyptus* oil is highly volatile (Lawler *et al.* 1999b), the detected oil decrease in dead leaves may actually have resulted from desiccation damage to oil storage organs such as oil glands (Fahn 1979; King *et al.* 2004), while storage of phenolics in the vacuole (Kutchan 2005; Wink 2010) remained secure. While we are unable to identify the mechanism which led to decreased oil content in leaves of plants in the 0% water treatment, we can confirm that the oil content which remained was still substantial (Fig. 1e). Retention of phenolic concentrations and 65% of the total oil concentrations in dead juvenile *E. globulus* leaves suggests ecological roles of these PSMs long after plant death (Whitham *et al.* 2012). For example, PSMs in juvenile *E. globulus* leaf litter could influence litter decomposition (Ushio *et al.* 2013), invertebrate communities (Barbour *et al.* 2009a) and flammability (Ormeño *et al.* 2009) after senescence from the plant. However, the longevity of PSM effects in leaf litter would depend on litter decomposition rates in local microclimates (Grime *et al.* 1996; Cornelissen *et al.* 1999).

Our data demonstrate that juvenile *E. globulus* PSM concentrations are highly stable during moderate to severe water deficit, and yet these results contrast with findings of earlier experiments (Chapter 2). It was previously reported that a 50% and 75% reduction in water availability decreased *E. globulus* TP concentrations (Chapter 2), yet here TP concentrations remained stable at these levels of water deficit (Table 2). While this discrepancy may be explained by differences in leaf sampling (all fully-expanded leaves of a plant [Chapter 2] compared to only the newest eight fully-expanded leaf pairs), these contrasting results suggest additional complexity in the impacts of experimental environment, experimental treatments and sampling methods on results. Compared to the present study, the experiment in Chapter 2 used different glasshouse facilities, seed provenances, treatment application techniques and fewer individual plants.

Overall, responses of PSM concentrations to water deficit vary between species and experiments (Miles *et al.* 1982; Doran & Bell 1994; Leicach *et al.* 2010; Pizarro & Bisigato 2010; Li *et al.* 2011; Yadav *et al.* 2014), and this was a partial catalyst for undertaking the work presented here. Contrasting and inconsistent changes to PSM concentrations have also been described resulting from experimental application of other environmental factors, including elevated CO_2 and ozone (O_3) (review by Lindroth 2010), UV light and temperature (review by Bidart-Bouzat & Imeh-Nathaniel 2008). These reviewers suggested that only further experimentation into how interacting environmental factors affect multiple PSM compounds using a broad range of species and genotypes would advance our understanding. However, even this may prove difficult when investigating water deficit impacts on plants given the lack of standardised water deficit treatments and the variation in experimental techniques (e.g. numerous levels of water deficit/stress based on polyethylene glycol [PEG], water exclusion, field capacity, Ψ_{soil} or stomatal conductance [g_s]).

5.5.3 *Conclusion*

Juvenile *E. globulus* were able to tolerate being provided 50% less water than control plants lost/used through evapotranspiration without any evidence of water stress. Furthermore, these *E. globulus* retained concentrations of each PSM at levels comparable to control plants even when provided only 10% of control evapotranspired water. Even leaves of dead plants contained PSM concentrations similar to controls, except for a decrease in the total oil yield in these desiccated leaves. While the total oil yield decreased in dead juvenile *E. globulus*, we found no indication that any level of water deficit inhibited the biosynthesis of one group of PSMs more than another group. We conclude that juvenile *E. globulus* can tolerate periods of continuous but minimal water availability with negative effects on plant growth and changes to physiological traits, yet with minimal impact on foliar PSM concentrations. These PSM responses may differ from those observed when other

environmental conditions such as nutrient levels, light intensity and temperature are altered.

Chapter 6

General Discussion

Soil water deficit limits CO_2 acquisition, plant nutrient uptake and photosynthesis (McDowell *et al.* 2008), which reduces the within-plant carbon pool available for use in plant growth (Mokotedi 2010; Pinkard *et al.* 2011) and PSM biosynthesis (Miles *et al.* 1982; Gleadow & Woodrow 2002; Zhang *et al.* 2012). The effect of soil water deficit on PSM concentrations has received considerable attention in the literature, and evidence suggests that PSM responses to soil water deficit vary considerably among species, PSM classes and experiments (Chapter 1; section 1.4). Most previous research investigated responses using woody eudicot species, yet the water deficit level, treatment duration and method of treatment application varied among studies (Supplemental Tables S1 and S2). Such variation in plant responses to water deficit and variation in experimental methods (including plant age/developmental stage) make it difficult to generalise response patterns of PSMs. The experiments described in this thesis (chapters 2-5) attempted to find such generalisations in juveniles of two sympatric woody eudicot species (*Eucalyptus* spp.) by comprehensively investigating the effect of different levels and durations of water deficit on a number of physiological, morphological and chemical traits between species and among provenances within species. I also tested the effect of re-watering after water deficit on these same plant traits. I hypothesised that provenances from wet localities would experience greater levels of water stress, and that traits would be quantitatively altered to a greater degree by a particular level of water deficit compared to provenances from drier localities. I also hypothesised that re-watering would reverse the effects of water deficit, and based on the literature, that levels of terpenes would be more plastic in response to soil water deficit than the levels of phenols.

Three general response patterns are evident from this work (Chapters 2-5).

(1) The responses to water deficit and re-watering of all assayed physiological, morphological and chemical traits (except foliar ABA levels) were uniform between species and among provenances within each species, regardless of rainfall variation among locations. Concentrations of the targeted non-nitrogen containing PSMs in leaves at different developmental stages on individual plants also responded similarly to water deficit. Therefore, the overall responses of these plants to water deficit were consistent, regardless of species, provenance, geographic origin or leaf age.

(2) Soil water deficit generally increased foliar ABA levels and made Ψ_{leaf} increasingly negative, while reducing above-ground growth and leaf water content.

(3) Each of the assayed PSM classes responded uniquely to water deficit, yet there were also inconsistent responses of the same chemical trait to water deficit between experiments. I will now discuss each of these three trends in turn.

Both *Eucalyptus* species, and each provenance within a species, responded similarly to all water deficit treatments and re-watering (except foliar ABA levels, see below). The responses were consistent among provenances and species regardless of whether the response was a quantitative trait change (increase or decrease), or if no effect of water deficit was detected. Furthermore, these responses occurred uniformly across species and provenances regardless of genetic-based quantitative variation in the constitutive level of each trait. The main four localities (each representing a genetically distinct provenance within each species) were selected for these experiments based on maximising climatic variability among localities, and for the variation in mature *E. globulus* drought tolerance among provenances (Dutkowski & Potts 2012). As such, the uniformity of responses to water deficit and re-watering among eucalypts from these localities was unexpected, especially given the variation in rainfall patterns among localities (Table 1 of

Chapter 3). The overwhelming uniformity of responses presented in this thesis may reflect genetic-based limitations to trait plasticity among provenances (discussed below). The only trait in which the response to water deficit varied between provenances was foliar ABA levels, and interestingly the response pattern was similar for both *Eucalyptus* species (Chapter 3). Foliar ABA signals partial or full stomatal closure depending on the foliar ABA level, and stomatal closure reduces plant water loss via transpiration and limits plant water stress (Tardieu & Simonneau 1998; Bauer *et al.* 2013; McAdam & Brodribb 2014). Given this, I hypothesised that eucalypts from the two drier localities (Queens Domain and St Helens) would have advanced stomatal control (i.e. foliar ABA levels would be higher during water deficit) as an adaptation to avoid water stress during the more frequent natural water deficit at these locations compared to wetter locations (King Island and Southern Tasmania). However, in contrast to my original hypothesis, the two locations in which foliar ABA levels were very high during water deficit (Southern Tasmania [wetter] and St Helens [drier]) were not both from dry locations. Recently published work using *Banksia* (Cochrane *et al.* 2014) and *Adansonia* (Bouda *et al.* 2014) populations also found that although variation in responses to water deficit varied among populations within a species, there was no link to rainfall patterns at site of origin. Therefore, the environmental factors at origin leading to the observed variation in foliar ABA responses among provenances are yet undefined.

The second overall response trend identified in this thesis was the reduction of above-ground growth and leaf water content, and increased leaf ABA levels and $-\Psi_{leaf}$. These plant responses to soil water deficit commonly occur (Chaves *et al.* 2003; McDowell *et al.* 2008; Brodribb & Cochard 2009; Choat *et al.* 2012). As such, these traits are important as they are fundamental indicators of plant water stress. Changes to these traits were expected during water deficit, and hence I was interested primarily in the magnitude of quantitative change to these traits as a measure of treatment effectiveness. Other physiological traits are commonly measured to assess plant water stress, including stomatal conductance (g_s) and assimilation rates (A)

(Costa e Silva *et al.* 2004; Blackman *et al.* 2009; Brodribb & Cochard 2009; Héroult *et al.* 2013; McAdam & Brodribb 2014). Given the large number of plants I grew simultaneously (>820 plants), it was not practical to use a portable infrared gas analyser (IRGA) to assess g_s and A at any one time across species, provenances and treatments. Instead, I took quantitative changes to growth, leaf water content, foliar ABA levels and Ψ_{leaf} as confirmation that the levels of water deficit (treatments) I provided were having a physiological impact on the plants (i.e. causing water stress). This method was successful, as only the 50% water deficit treatment in chapter 4 appears to have been less effective than planned. In chapter 4, a 50% reduction in water availability did not increase foliar ABA levels or limit growth (above-ground biomass), yet this treatment did lead to a more negative Ψ_{leaf} and decreased leaf water content. This was the only experiment where I used *E. viminalis* alone, and while no variation in tolerance to water deficit was detected between the *Eucalyptus* species in Chapters 2-3, perhaps a 50% reduction in available water (the same treatment used in Chapters 2, 4-5) had less of an impact on normal *E. viminalis* function than it did on *E. globulus*. With regard to the measurement of plant growth, perhaps a different technique would have led to the detection of changes in growth due to water deficit in Chapter 4. For example, while quantifying above-ground biomass (Chapters 3-5) was an improvement over measuring plant height (Chapter 2), counting node numbers at the beginning and end of treatments in addition to quantifying biomass would have enabled better assessment of plant growth *during* the treatments. Nevertheless, throughout the four experiments the fairly consistent changes to growth, leaf water content, foliar ABA levels and Ψ_{leaf} due to the treatments provided good evidence of water stress levels (even though the actual quantitative change to each trait varied between experiments), which provided a basis to interpret the more variable effects of water deficit and re-watering on PSM concentrations.

The third overall trend found throughout this thesis is that quantitative responses to water deficit and re-watering of the assayed terpenes and phenols

differed among PSM classes, among individual compounds and among experiments. Thus, it appears that the responses of the leaf secondary metabolites I quantified are idiosyncratic. Given this response variation, there is difficulty in summarising the response of each assayed PSM class/compound to soil water deficit. These inconsistent responses are likely to result from variation in experimental design among experiments (number of plants, species used), the impact that experimental design had on statistical significance (effects of replication on statistical power), the use of open-pollinated seed (unknown paternal genotype of each juvenile eucalypt) and the stress levels induced by each water deficit treatment (10 different levels of water deficit in total). In order to summarise the influences of soil water deficit and re-watering on concentrations of PSMs (specific terpenes and phenols) across species and provenances, I have focused only on the changes to PSM concentrations found to be statistically significant due to 50% water deficit, even though these were inconsistent among experiments (Fig. 1). The 50% water treatment was the only common level of water deficit across all four experiments, and most significant changes to PSM concentrations occurred during this treatment. The only exceptions are reduced total oil concentrations in 0% water plants (Chapter 5) and reduced total phenolic concentrations during 25% water (Chapter 2). In summarising responses (Fig. 1), the 50% water deficit increased the total oil concentrations (Chapter 4), and re-watering decreased total oil to levels comparable with controls (Chapter 4). In contrast, FPC concentrations decreased during water deficit (Chapter 3), and these low concentrations were not influenced by re-watering (Chapter 3). Total phenolic concentrations decreased (Chapter 2) and condensed tannin concentrations increased during water deficit (Chapter 3), however, total phenolic concentrations also decreased during re-watering (Chapter 3) while concentrations of condensed tannins returned to control levels (Chapter 3). For the complete summary of statistically significant and non-significant changes to plant traits as a result of 50% soil water deficit refer to Supplemental Table S7.

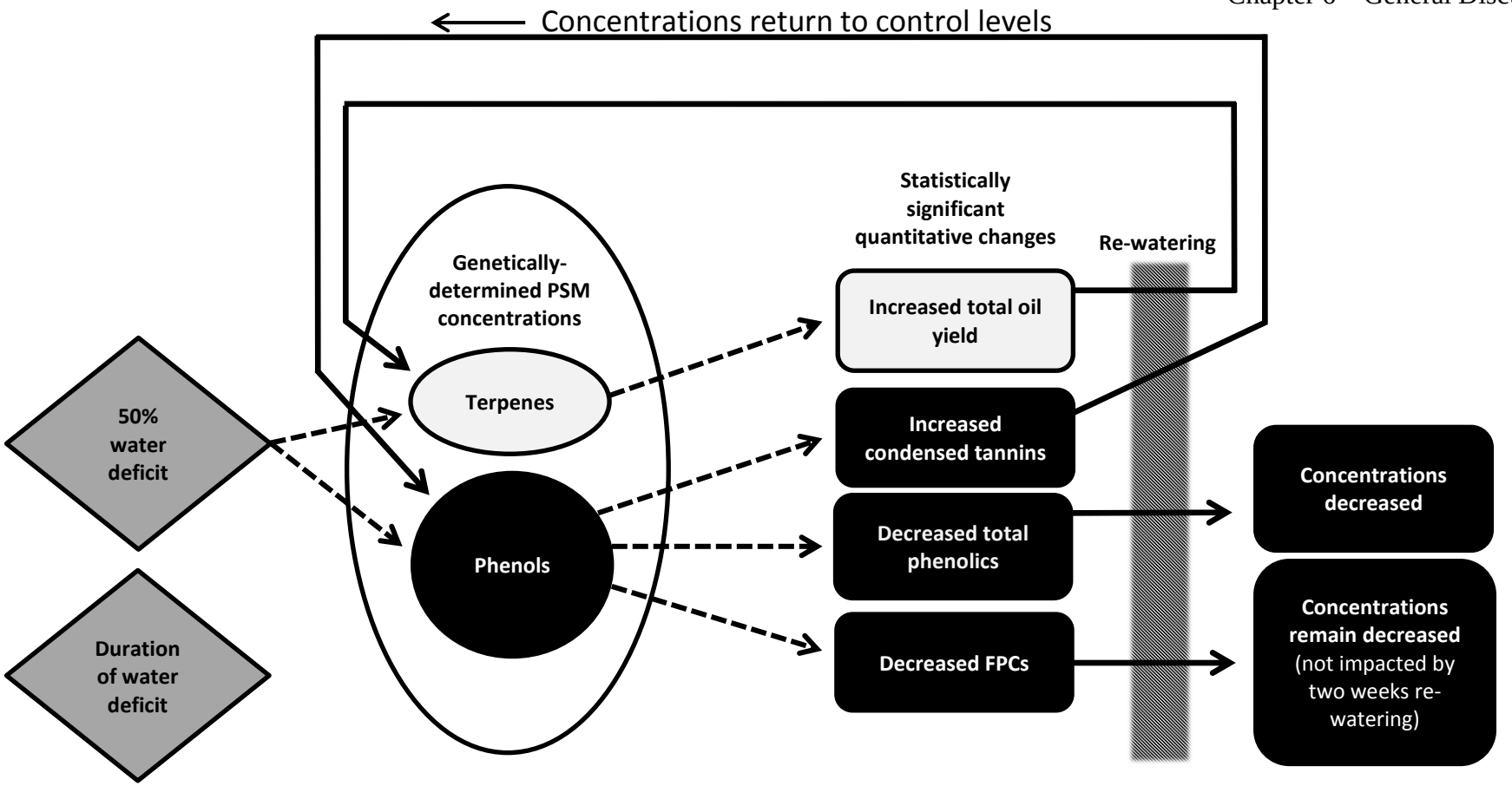

Figure 1. The statistically significant effects of decreased water availability (50% of the water used/lost from control pots via evapotranspiration), the duration of water deficit, and re-watering after water deficit on genetic-based plant secondary metabolite (PSM) concentrations of juvenile *Eucalyptus globulus* and *E. viminalis* leaves. Broken arrows follow the effect of water deficit on PSM classes (ovals), solid arrows follow the effect of re-watering on PSM concentrations. Grey shapes indicate environmental effects, black shapes indicate phenols and white shapes indicate terpenes. The duration of water deficit had no effect on PSM concentrations. Significant results taken from Chapters 2-5. Re-watering refers to the application of water to field capacity for 12-14 days to plants which had previously been subjected to a 50% water availability treatment. Only select terpenes and phenols were quantified.

The variation in PSM responses to water deficit among chapters (chapters 2-5) and traits (Supplemental Table S7) make it difficult to discuss responses within a framework of plant defence theory. At first glance, one could surmise that there was a resource-based trade-off (Agrawal 2011b) between growth/phenolic concentrations (excluding condensed tannins) and terpenes/condensed tannin concentrations during water deficit (Figure 1; Supplemental Table S7). In this scenario the trade-off is caused by limited water availability, where terpene concentrations increase and provide a benefit under water deficit, yet the trade-off (cost) is that phenol concentrations decrease along with the benefit that phenols provide. However, given the variation in responses among chapters, there is scant support for this scenario. One factor that may have masked a consistent trade-off among different PSM concentrations is variation in plant vigour within a species (Agrawal 2011a). Vigorous plants use more resources, grow larger and have higher concentrations of PSMs (Agrawal 2011a). In this situation, a clear trade-off between growth and the concentration of different PSMs may be identified if each PSM concentration was standardised against a measure of plant vigour (Hare *et al.* 2003). At the beginning of this thesis I proposed that responses to water deficit may follow the growth-differentiation balance hypothesis (GDBH) of Herms and Mattson (1992). The experimental design of chapter 5 in particular was ideal for testing this hypothesis, yet I found no indication that PSM concentrations increased while growth decreased under mild water deficit, and both growth and PSM concentrations did not decrease under severe water deficit. As such, this work has provided very little evidence of a trade-off (cost) to growth and PSM synthesis during water deficit, assuming that there was a trade-off amongst the measured plant traits under those glasshouse conditions (Agrawal 2007).

A literature review (chapter 1) suggested that terpene concentrations in woody eudicot leaves (including eucalypts) are more plastic in response to water deficit than phenol concentrations, and yet I found the opposite. However, with the inclusion of previous published studies also using water limited *Eucalyptus* (Miles *et*

al. 1982; Doran & Bell 1994; Gleadow & Woodrow 2002; Leicach *et al.* 2010), there appears to be no difference in plasticity levels between terpenes and phenols in eucalypts (Fig. 2). Across studies, terpene concentrations changed 25-37% of the time, and phenol concentrations (excluding chlorogenic acid as only I have quantified this compound) changed 29-38% of the time due to soil water deficit (Fig. 2). While levels of plasticity appear similar between these two major PSM groups, the type of quantitative response seems to differ among compounds (Fig. 2). For example, α-pinene and condensed tannin concentrations in *Eucalyptus* leaves appear most likely to increase during water deficit, whereas concentrations of total phenolics and FPCs appear most likely to decrease (Fig. 2). However, most of the time (≥50% of reports) the concentrations of these PSMs in *Eucalyptus* leaves are not influenced by soil water deficit.

Overall, different conclusions regarding trait plasticity and the common type of response (increased or decreased concentrations) of PSM concentrations to water deficit can be formed when using either the statistically significant finding of this thesis (such as those at 50% water deficit in Fig. 1), all individual quantitative trait changes (significant and non-significant; Supplemental Table S7), all work on eucalypts (Fig. 2), or all studies across plant taxa (Fig. 1 in Chapter 1). Few studies have tested water deficit impacts on PSM concentrations of *Eucalyptus* (largest number of studies is eight per compound, with six of those findings from this thesis; Fig. 2). This is an insufficient number of studies to form unambiguous response conclusions of this genus, let alone of an individual species. Many more studies are required across *Eucalyptus* species to ascertain true PSM response trends during water deficit.

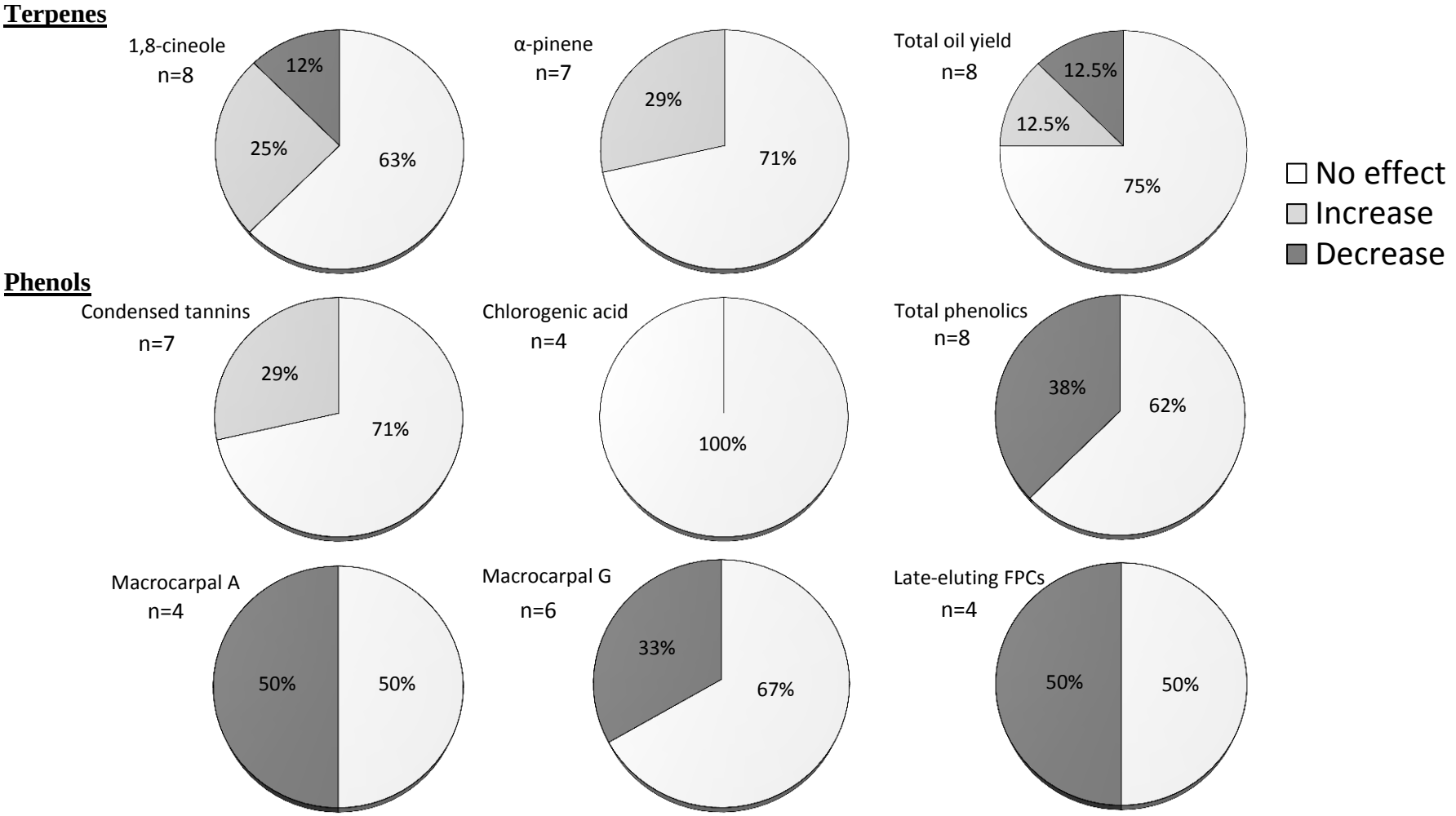

Figure 2. The effect of water deficit on concentrations of select terpenes and phenols in *Eucalyptus* leaves. Responses include *Eucalyptus* data published by other authors (Supplemental Tables S1 & S2) combined with significant results taken from experimental chapters 2-5 of this thesis. Percentage is each wedge indicates the proportion of all reports that the response was reported. The total number of reports (*n*) are given for each individual PSM trait.

The experiments described in this thesis enabled a comparison of genetic-based PSM responses among juvenile *E. globulus* and *E. viminalis* from four different localities to a continuous sub-optimal level of water availability in a common environment. From this work, inferences can be made as to how altered concentrations of these particular PSMs during drought would impact ecological interactions and processes. My results show that when water deficit changed concentrations of bulk phenol groups, the condensed tannin concentrations increased by 14–87% and the total phenolics decreased by 15–18%. An estimate of the ecological effect of these changes can be garnered from the literature. For example, a reduction in condensed tannin concentration of 19% in *Salix* in response to water deficit had no effect on blue leaf beetle (*Phratora vulgatissima*) performance (Glynn *et al.* 2004). Likewise, a 48% reduction in the concentration of phenolics in *E. camaldulensis* leaves during water deficit had no impact on the rate of development of *Pieris rapae* caterpillars and *Paropsis atomaria* larvae (Miles *et al.* 1982). The only evidence of soil water deficit induced changes in bulk phenol concentrations having an impact on associated biota was reported by Shure *et al.* (1998). Shure *et al.* (1998) found that natural dry periods lowered condensed tannin and total phenolic concentrations by 27-48% in *Quercus* and *Acer* leaves, which decreased leaf damage by invertebrate herbivores and fungal pathogens in conjunction with lower foliar water and N content. Given that total phenolics and condensed tannins are bulk assays of an unknown number of un-identified compounds, the specific compounds in total phenolic and condensed tannin extracts of *Quercus* and *Acer* may differ from those in *Eucalyptus* extracts. As such, the ecological effect of quantitative changes to these bulk PSMs may differ among species depending on whether the effect is a result of total phenol/tannin concentrations or the concentrations of particular compounds. With respect to FPC concentrations, I found that water deficit decreased the concentration of macrocarpals by 9-18% (Chapter 3), yet nearly a two-fold difference in macrocarpal G concentration is required to alter *Eucalyptus* leaf intake by common ringtail possums (Lawler *et al.* 1998). A similar magnitude of quantitative variation (c. 80-90%) in sideroxylonal A concentrations is necessary to influence food intake by common brushtail possums (Wallis *et al.* 2002). As such,

the relatively minor changes to phenol and FPC concentrations in juvenile *E. globulus* and *E. viminalis* reported in this thesis would likely have limited impacts on biotic associations such as herbivory.

While the above ecological inferences can be made, the results of these experiments are not directly comparable to the effects of natural drought events on the same plant chemical traits in field locations. I thoroughly tested the effects of water deficit on potted juvenile plants of two *Eucalyptus* species grown in potting mix in a climatically controlled and common environment by providing continuous yet sub-optimal levels of water. The potting mix had a higher nutrient content than typical Australian soil, and it likely also had a different water holding capacity. My work eliminates the effect of environment (except for the applied treatments) and focuses on genetic-based responses to unnatural water deficit in a uniform substrate. Meteorological drought differs dramatically from my experimental treatments, as cessation of rainfall and limited access to soil water causes severe water stress and even plant death (Bréda *et al.* 2006; Carnicer *et al.* 2011; Anderegg *et al.* 2013; Floyd *et al.* 2015). In order to test the influence of future drought periods on plant chemistry and PSM mediated interactions in a natural situation, an assessment of provenance responses to natural drought in each native location would be required (e.g. with local soil type, nutrient level, irradiation levels, air humidity and supplemental watering of some plants as controls). Of course, it is then not possible to test genetic (G) or environmental (E) effects in isolation, with the droughted eucalypt phenotype resulting from the full $G + E + (G \times E)$ interaction. A compromise could be the incorporation of locality-specific conditions into glasshouse trials. Conducting similar experiments to those outlined here (Chapters 3-5) using soil collected from each locality would bridge the gap between glasshouse- and field-based experiments and improve our understanding of locality responses to water deficit, which may differ due to the water holding capacity and nutrient properties of the substrate. However, a negative effect of introducing local soil types

to this experimental design is increased plant numbers in order to test for soil effects as well as genetic (species, locality) and water treatment effects.

Future Directions

I was unable to detect intra-specific variation in the responses of PSMs (selected terpenes and phenols) to soil water deficit or re-watering. PSM plasticity appears highly conserved in juveniles of these two eucalypt species, and perhaps limited diversification of induced responses to water deficit has occurred among provenances. Each provenance used in this thesis was from south-eastern Australia, and environmental gradients exist among the localities (Table 1 in Chapter 3). The use of a more widely distributed species, or a species which occupies a wider ecological niche than *E. globulus* and *E. viminalis* would increase the likelihood of detecting intra-specific variation in PSM responses. For example, *E. camaldulensis* occurs over a wide geographic area, as it naturally occurs in all mainland Australian states (Butcher *et al.* 2009). While previous studies have investigated the effect of water deficit on PSM concentrations in *E. camaldulensis* (Doran & Bell 1994; Stone & Bacon 1994; Leicach *et al.* 2010), no study has tested responses among *E. camaldulensis* provenances. Even though *E. camaldulensis* occupies a narrow ecological niche (riverbanks; Butcher *et al.* 2009), the vast distance between populations (geographic isolation) and associated broad-scale climate variation may have led to diversifying selection (Yoder *et al.* 2010) and intra-specific variation in trait plasticity. Alternatively, intra-specific variation in PSM responses may be detected using species that occupy a broad range of environments, as diverse environments would likely maximise the variation in selection pressures on individual populations, and drive population adaptation to local conditions through diversifying selection.

With the exception of the extremely low levels of water availability used in Chapter 5 (20 - 0% control evapotranspiration), the level of water I provided plants (generally 50%) induced only mild water stress. In retrospect, the level of water deficit used in Chapters 3 and 4 could have been more severe. This water deficit level (50%) was selected based on the results of Chapter 2 and the common use of similar treatments in the literature (Supplemental Tables S1 and S2). The intention was that eucalypts should maintain carbon assimilation and water-bound nutrient uptake, so that the resources were available if PSM synthesis was upregulated during water deficit. From my results (Chapters 2 and 5) I estimate that 15-25% of evapotranspiration from control plants would be an appropriate level of water deficit in which to test the response of PSMs in eucalypts to water deficit. This amount of available water (15-25% evapotranspiration) should cause turgor loss, limit stomatal conductance and reduce plant growth as reported in Chapter 5, but also force plants to prioritise the use of very limited resources to growth or photoassimilates (Herms & Mattson 1992; McKinnon *et al.* 1998; Hale *et al.* 2005).

Along with a more severe level of water deficit, another consideration for further work is the plant developmental stage when water deficit is applied. I began all treatments when eucalypts were around 6 months of age, when eucalypts were juvenile plants (c. 50cm tall) rather than seedlings. The reason for beginning treatments when eucalypts were 6 months old was to ensure that substantial leaf biomass existed for the various chemical assays. In doing this, I created a situation where some leaves of each plant in Chapters 2-5 expanded prior to the treatment, and some expanded during the water deficit treatment. When harvesting the leaves in Chapters 3-5, I only sampled leaves which I believed had expanded during the treatment (youngest eight fully-expanded *E. globulus* leaf pairs or uppermost 50% of fully-expanded *E. viminalis* leaves). This method worked adequately for the sampling of leaves in Chapters 3 and 4 (only one level of water deficit = 50%), however, the *E. globulus* in Chapter 5 which received very low amounts of water (e.g. 10% of control evapotranspiration) did not grow after the treatment was applied.

This made it difficult to standardise the sampling of *E. globulus* leaves on control plants (which shed lower leaves during development) with plants in the 10% water treatment (which produced few new leaves once the treatment began). Commencing water deficit treatments while plants are at an early developmental stage would mean that all leaves expand during the treatment and can be harvested for analysis of plant responses. Water stress during early plant development may also trigger gene expression in the seedling which alters its development from an early stage (Chaves *et al.* 2003). Applying water deficit treatments to seedlings would provide a better indication of water deficit effects on seedling development during drought than beginning treatments when plants are established (6 months old).

The findings presented in this thesis represent responses of juvenile *E. globulus* and *E. viminalis* to levels of continuous water deficit, and may not reflect the responses of adult trees from these same provenances. Due to the distinct ontogenetic phases which many eucalypts transition through to reach adult form (Gras *et al.* 2005; Goodger *et al.* 2006; Loney *et al.* 2006a; Goodger *et al.* 2007; McArthur *et al.* 2010; Borzak *et al.* 2015), the responses of adult eucalypts from these provenances to water deficit need to be tested. Subjecting mature eucalypts to a continuous level of water deficit in the field may prove difficult. This difficulty arises from an inability of a researcher to completely control soil water content in a field situation, especially given the extensive root systems of mature eucalypt trees (Collison 2001; Eamus *et al.* 2002). Perhaps the combined use of sap flow meters, stomatal conductance and water potential data would provide some indication of water uptake and plant stress in common-garden grown adult eucalypts during drought. For comparison, neighbouring trees could be irrigated for use as controls (White *et al.* 1996; White *et al.* 1998). In doing so, the effect of natural drought on genetic-based physiological, morphological (new growth) and chemical traits in mature eucalypts could be tested and compared to results presented in this thesis using juvenile eucalypts.

The experimental chapters of this thesis tested the effects of water deficit on concentrations of a subset of non-nitrogen containing PSMs, by quantifying a number of phenol and terpene PSM groups/compounds. The chosen terpenes and phenols were selected due to their abundance in foliage of these two species, their quantification in relation to water deficit in previous work (Supplemental Tables S1 and S2), and their known ecological roles (section 1.2.3). In doing so, many low abundance terpenes, individual phenols, and all nitrogen-containing PSMs were ignored. While low abundance terpenes may be of little ecological importance simply due to low concentrations, there is enormous opportunity for future studies to quantify the effect of water deficit on individual phenols, as well as nitrogen-containing compounds. Previous studies have quantified the effect of water deficit on concentrations of cyanogenic glycosides in eucalypts (Gleadow & Woodrow 2002; Woodrow *et al.* 2002), yet limited work has focused on other classes such as the highly diverse alkaloids. However, recent work has suggested that continued quantification of individual PSMs may be of less ecological and evolutionary importance than quantifying overall combinations of defensive chemical traits which form the defence syndrome of a species (Agrawal 2011a). In this case, the response of a plant defence syndrome to water deficit may be more informative for understanding the ecological ramifications of drought.

The final limitation of this research which I feel I could have explored further is the effect of water deficit on below-ground plant traits. Quantifying root traits (e.g. mass, length and architecture) would have been a valuable addition to this work, and would have provided a more complete overview of treatment effects on the juvenile eucalypts. However, below-ground sampling was not undertaken due to the extra time required, the research focus on leaf traits, and the large number of leaf samples which required processing without the addition of root samples. Outside the scope of this thesis but of great interest would be the effect of water deficit on root PSM concentrations, and the subsequent effect of altered root PSM levels on soil food webs and nutrient cycling. The integration of water deficit, PSM leachate from leaf

litter, plant root PSM levels and soil food webs would be an invaluable addition to our knowledge of drought impacts on the *Eucalyptus* system.

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Appendices

Supplemental Table S1. The influence of soil water deficit on total oil concentrations or individual terpenes in a range of plant species[1]

Compound/group	Species	Plant form	Treatment	Effect	% change	Reference
1,8-Cineole	***Eucalyptus camaldulensis***	**Woody eudicot**	**Rain exclusion**	**Decrease**	**34**	**(Doran & Bell 1994)**
1,8-Cineole	***Eucalyptus camaldulensis***	**Woody eudicot**	**33**	**Increase**	**28.3**	**(Leicach *et al.* 2010)**
1,8-Cineole	*Salvia officinalis*	Woody eudicot	25	Increase	201	(Bettaieb *et al.* 2009)
1,8-Cineole	*Salvia officinalis*	Woody eudicot	33	Increase	33	(Nowak *et al.* 2010)
1,8-Cineole	*Salvia officinalis*	Woody eudicot	50	Increase	417	(Bettaieb *et al.* 2009)
Limonene	***Eucalyptus camaldulensis***	**Woody eudicot**	**33**	**Decrease**	**55**	**(Leicach *et al.* 2010)**
Limonene	*Pinus halepensis*	Conifer	33	No effect	.	(Blanch *et al.* 2009)
Limonene	*Quercus ilex*	Woody eudicot	33	No effect	.	(Blanch *et al.* 2009)
Total oil yield	*Artemisia annua*	Herbaceous eudicot	40	Decrease	56	(Yadav *et al.* 2014)
Total oil yield	*Artemisia annua*	Herbaceous eudicot	60	Decrease	22	(Yadav *et al.* 2014)
Total oil yield	***Eucalyptus camaldulensis***	**Woody eudicot**	**Rain exclusion**	**Decrease**	**34**	**(Doran & Bell 1994)**
Total oil yield	***Eucalyptus camaldulensis***	**Woody eudicot**	**33**	**No effect**	**.**	**(Leicach *et al.* 2010)**
Total oil yield	*Lupinus albus*	Herbaceous eudicot	Water withheld	No effect	.	(Carvalho *et al.* 2004)
Total oil yield	*Lupinus mutabilis*	Herbaceous eudicot	Water withheld	No effect	.	(Carvalho *et al.* 2004)
Total oil yield	*Pinus halepensis*	Conifer	33	Increase	54	(Blanch *et al.* 2009)
Total oil yield	*Quercus ilex*	Woody eudicot	33	Increase	119	(Blanch *et al.* 2009)
Total oil yield	*Salvia officinalis*	Woody eudicot	25	Increase	259	(Bettaieb *et al.* 2009)
Total oil yield	*Salvia officinalis*	Woody eudicot	50	Increase	454	(Bettaieb *et al.* 2009)
Total terpenes	*Pinus halepensis*	Conifer	Rain exclusion	Seasonally dependant	.	(Llusià *et al.* 2006)
Total terpenes	*Pistacia lentiscus*	Woody eudicot	Rain exclusion	Seasonally dependant	.	(Llusià *et al.* 2006)
Total terpenes	*Rosmarinus officinalis*	Woody eudicot	Rain exclusion	Seasonally dependant	.	(Llusià *et al.* 2006)
α-Pinene	*Cistus albidus*	Woody eudicot	Water withheld	No effect	.	(Llusià & Peñuelas 1998)
α-Pinene	***Eucalyptus camaldulensis***	**Woody eudicot**	**33**	**Increase**	**0.74**	**(Leicach *et al.* 2010)**
α-Pinene	*Pinus halepensis*	Conifer	33	No effect	.	(Blanch *et al.* 2009)
α-Pinene	*Pinus halepensis*	Conifer	Water withheld	Increase	46	(Llusià & Peñuelas 1998)
α-Pinene	*Pistacia lentiscus*	Woody eudicot	Water withheld	Increase	56	(Llusià & Peñuelas 1998)
α-Pinene	*Quercus ilex*	Woody eudicot	33	Increase	83	(Blanch *et al.* 2009)
α-Pinene	*Salvia officinalis*	Woody eudicot	25	Increase	1471	(Bettaieb *et al.* 2009)
α-Pinene	*Salvia officinalis*	Woody eudicot	50	Increase	2453	(Bettaieb *et al.* 2009)
Monoterpenes	*Salvia officinalis*	Woody eudicot		Increase	33	(Nowak *et al.* 2010)

[1] *Treatment* indicates the percentage of water provided to plants based on the water used/provided to controls (100% water in each respective experiment). *Water withheld* indicates that plants were not watered for a period of time or until death. *Rain exclusion* indicates that direct rainfall was withheld from plants, yet ground-water may have been available. *% change* is the effect size of water deficit in comparison to PSM concentrations in controls. Reports using eucalypt species are highlighted. *No effect* indicates no statistically significant effect of water deficit on that trait was detected in that study.

Supplemental Table S2. The influence of soil water deficit on foliar phenol concentrations in a range of plant species[2]

Compound/group	Species	Plant form	Treatment	Effect	% change	Reference
Anthocyanins	*Triticum aestivum*	Monocot	PEG (-0.49 MPa)	Increase	129	(Ma *et al.* 2014)
Chlorogenic acid	*Crataegus spp.*	Woody eudicot	Water withheld	Increase	58	(Kirakosyan *et al.* 2004)
Chlorogenic acid	*Helianthus annuus*	Herbaceous eudicot	NaCl addition	Increase	600	(del Moral 1972)
Chlorogenic acid	*Salix viminalis* x *S. dasyclados*	Woody eudicot	55	No effect	.	(Glynn *et al.* 2004)
Cinnamic acid	*Salix viminalis* x *S. dasyclados*	Woody eudicot	55	No effect	.	(Glynn *et al.* 2004)
Condensed tannins	*Acer rubrum*	Woody eudicot	Decreased rainfall	Increase	27	(Shure *et al.* 1998)
Condensed tannins	*Acer saccharum*	Woody eudicot	Water withheld	No effect	.	(Roth *et al.* 1997)
Condensed tannins	*Bougainvillea spinosa*	Woody eudicot	33	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Bougainvillea spinosa*	Woody eudicot	47	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Bougainvillea spinosa*	Woody eudicot	60	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Bougainvillea spinosa*	Woody eudicot	73	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Ceratonia siliqua*	Woody eudicot	~	Increase	~	(Kouki & Manetas 2002)
Condensed tannins	*Chuquiraga erinacea*	Woody eudicot	33	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Chuquiraga erinacea*	Woody eudicot	47	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Chuquiraga erinacea*	Woody eudicot	60	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Chuquiraga erinacea*	Woody eudicot	73	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	***Eucalyptus cladocalyx***	**Woody eudicot**	**25**	**No effect**	**.**	**(Gleadow & Woodrow 2002)**
Condensed tannins	*Grindelia chiloensis*	Herbaceous eudicot	33	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Grindelia chiloensis*	Herbaceous eudicot	47	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Grindelia chiloensis*	Herbaceous eudicot	60	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Grindelia chiloensis*	Herbaceous eudicot	73	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Jarava speciosa*	Monocot	33	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Jarava speciosa*	Monocot	47	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Jarava speciosa*	Monocot	60	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Jarava speciosa*	Monocot	73	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Larrea divaricata*	Woody eudicot	33	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Larrea divaricata*	Woody eudicot	47	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Larrea divaricata*	Woody eudicot	60	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Larrea divaricata*	Woody eudicot	73	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Lotus corniculatus (cv Leo)*	Herbaceous eudicot	60	Decrease	15	(Carter *et al.* 1999)
Condensed tannins	*Populus tremuloides*	Woody eudicot	Water withheld	No effect	.	(Roth *et al.* 1997)
Condensed tannins	*Prosopis alpataco*	Woody eudicot	33	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Prosopis alpataco*	Woody eudicot	47	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Prosopis alpataco*	Woody eudicot	60	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Prosopis alpataco*	Woody eudicot	73	No effect	.	(Pizarro & Bisigato 2010)
Condensed tannins	*Quercus alba*	Woody eudicot	Decreased rainfall	Decrease	31	(Shure *et al.* 1998)
Condensed tannins	*Quercus prinus*	Woody eudicot	Decreased rainfall	No effect	.	(Shure *et al.* 1998)
Condensed tannins	*Salix viminalis* x *S. dasyclados*	Woody eudicot	55	Decrease	19	(Glynn *et al.* 2004)
Flavonoids	*Salix viminalis* x *S. dasyclados*	Woody eudicot	55	No effect	.	(Glynn *et al.* 2004)

Supplemental Table S2 (continued)

Compound/group	Species	Plant form	Treatment	Effect	% change	Reference
Flavonoids	*Triticum aestivum*	Monocot	PEG (-0.49 Mpa)	Increase	54.5	(Ma *et al.* 2014)
Phenolic glycosides	*Populus nigra*	Woody eudicot	Decreased PMMT	Increase	89	(Hale *et al.* 2005)
Salicylates	*Salix viminalis* x *S. dasyclados*	Woody eudicot	55	No effect	.	(Glynn *et al.* 2004)
Total phenolics	*Acer rubrum*	Woody eudicot	Decreased rainfall	Decrease	43	(Shure *et al.* 1998)
Total phenolics	*Apera spica-venti*	Monocot	25	No effect	.	(Sakalauskiene *et al.* 2013)
Total phenolics	*Artemisia annua*	Herbaceous eudicot	40	Increase	960.6	(Yadav *et al.* 2014)
Total phenolics	*Artemisia annua*	Herbaceous eudicot	60	Increase	300	(Yadav *et al.* 2014)
Total phenolics	*Bougainvillea spinosa*	Woody eudicot	33	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Bougainvillea spinosa*	Woody eudicot	47	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Bougainvillea spinosa*	Woody eudicot	60	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Bougainvillea spinosa*	Woody eudicot	73	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Ceratonia siliqua*	Woody eudicot	~	Increase	~	(Kouki & Manetas 2002)
Total phenolics	*Chuquiraga erinacea*	Woody eudicot	33	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Chuquiraga erinacea*	Woody eudicot	47	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Chuquiraga erinacea*	Woody eudicot	60	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Chuquiraga erinacea*	Woody eudicot	73	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Desi ajwain*	Herbaceous eudicot	60	Increase	99	(Azhar *et al.* 2011)
Total phenolics	*Desi ajwain*	Herbaceous eudicot	80	Increase	77	(Azhar *et al.* 2011)
Total phenolics	*Erica multiflora*	Woody eudicot	75-80 (in Summer)	Increase	25	(Nogués *et al.* 2012)
Total phenolics	*Erica multiflora*	Woody eudicot	75-80 (in Spring)	No effect	.	(Nogués *et al.* 2012)
Total phenolics	***Eucalyptus camaldulensis***	**Woody eudicot**	**Water withheld**	**Decrease**	**48**	**(Miles *et al.* 1982)**
Total phenolics	***Eucalyptus cladocalyx***	**Woody eudicot**	**25**	**No effect**	**.**	**(Gleadow & Woodrow 2002)**
Total phenolics	*Grindelia chiloensis*	Herbaceous eudicot	47	Decrease	39	(Pizarro & Bisigato 2010)
Total phenolics	*Grindelia chiloensis*	Herbaceous eudicot	33	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Grindelia chiloensis*	Herbaceous eudicot	60	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Grindelia chiloensis*	Herbaceous eudicot	73	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Hypericum polyanthemum*	Herbaceous eudicot	~	Increase	~	(de Matos Nunes *et al.* 2014)
Total phenolics	*Jarava speciosa*	Monocot	33	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Jarava speciosa*	Monocot	47	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Jarava speciosa*	Monocot	60	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Jarava speciosa*	Monocot	73	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Labisia pumila* var. *alta*	Woody eudicot	25	Increase	112	(Jaafar *et al.* 2012)
Total phenolics	*Labisia pumila* var. *alta*	Woody eudicot	50	Increase	116	(Jaafar *et al.* 2012)
Total phenolics	*Labisia pumila* var. *alta*	Woody eudicot	75	Increase	52	(Jaafar *et al.* 2012)
Total phenolics	*Labisia pumila* var. *pumila*	Woody eudicot	25	Increase	132	(Jaafar *et al.* 2012)
Total phenolics	*Labisia pumila* var. *pumila*	Woody eudicot	50	Increase	139	(Jaafar *et al.* 2012)
Total phenolics	*Labisia pumila* var. *pumila*	Woody eudicot	75	Increase	61	(Jaafar *et al.* 2012)
Total phenolics	*Larrea divaricata*	Woody eudicot	33	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Larrea divaricata*	Woody eudicot	47	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Larrea divaricata*	Woody eudicot	60	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Larrea divaricata*	Woody eudicot	73	No effect	.	(Pizarro & Bisigato 2010)

Supplemental Table S2 (continued)

Compound/group	Species	Plant form	Treatment	Effect	% change	Reference
Total phenolics	*Phaseolus lunatus*	Woody eudicot	12.5	No effect	.	(Ballhorn *et al.* 2011)
Total phenolics	*Phaseolus lunatus*	Woody eudicot	25	No effect	.	(Ballhorn *et al.* 2011)
Total phenolics	*Populus nigra* (5 clones)	Woody eudicot	Summer drought	Increase	varies	(Fernàndez-Martínez *et al.* 2013)
Total phenolics	*Prosopis alpataco*	Woody eudicot	47	Increase	44	(Pizarro & Bisigato 2010)
Total phenolics	*Prosopis alpataco*	Woody eudicot	60	Increase	21	(Pizarro & Bisigato 2010)
Total phenolics	*Prosopis alpataco*	Woody eudicot	33	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Prosopis alpataco*	Woody eudicot	73	No effect	.	(Pizarro & Bisigato 2010)
Total phenolics	*Quercus alba*	Woody eudicot	Decreased rainfall	Decrease	50	(Shure *et al.* 1998)
Total phenolics	*Quercus prinus*	Woody eudicot	Decreased rainfall	Decrease	48	(Shure *et al.* 1998)
Total phenolics	*Stipa grandis*	Monocot	15	Decrease	15	(Chen *et al.* 2013)
Total phenolics	*Stipa krylovii*	Monocot	15	Increase	30	(Chen *et al.* 2013)
Total phenolics	*Triticum aestivum*	Monocot	PEG (-0.49 Mpa)	Increase	96.5	(Ma *et al.* 2014)
Total phenolics	*Triticum aestivum* (FD-83)	Monocot	15	Increase	16	(Hameed *et al.* 2013)
Total phenolics	*Triticum aestivum* (Nesser)	Monocot	15	Increase	27	(Hameed *et al.* 2013)
Total phenolics	*Zea mays (Ankora - susceptible)*	Monocot	50	Increase	varies	(Hura *et al.* 2008)
Total phenolics	*Zea mays (Nova - moderate)*	Monocot	50	Increase	varies	(Hura *et al.* 2008)
Total phenolics	*Zea mays (Tina - tolerant)*	Monocot	50	Decrease	varies	(Hura *et al.* 2008)

[2] *Treatment* indicates the percentage of water provided to plants based on the water used/provided to controls (100% water in each respective experiment). *Water withheld* indicates that plants were not watered for a period of time or until death. *Rain exclusion* indicates that direct rainfall was withheld from plants, yet ground-water may have been available. *Decreased rainfall* indicates naturally reduced rainfall compared to other years/sites. *PEG* Polyethylene glycol used to bind water molecules and make water unusable by the plant, thereby simulating soil water deficit of certain soil water potential. *Decreased PMMT* indicates that water was applied when the potting medium moisture tension (PMMT) reached a desired deficit level. *% change* is the change in concentration due to water deficit in comparison to PSM concentrations in controls. All reports using *Eucalyptus* are highlighted. *No effect* indicates no statistically significant effect of water deficit on that trait was detected in that study.

Supplemental Table S3. Least squares mean (±SE) values of traits from juvenile ***Eucalyptus globulus*** grouped localities (King island, Southern Tasmania, Queens Domain, St Helens), sampling period (Harvest1 or two weeks after at Harvest 2) and treatments (control, water limited/recovery)[3]

	King Island				**Southern Tasmania**				**Queens Domain**				**St Helens**			
	Harvest 1		**Harvest 2**		**Harvest 1**		**Harvest 2**		**Harvest 1**		**Harvest 2**		**Harvest 1**		**Harvest 2**	
	Control	*Water limited*	*Control*	*Recovery*	*Control*	*Water limited*	*Control*	*Recovery*	*Control*	*Water limited*	*Control*	*Recovery*	*Control*	*Water limited*	*Control*	*Recovery*
LMA	59.9(3.2)	62.2(3.2)	59.7(3.1)	52.1(3.3)	68.2(3.2)	68.7(3.1)	59.5(3.1)	54.9(3.1)	53.9(3.1)	66.0(3.2)	57.2(3.6	53.1(2.9)	54.7(3.1)	68.3(3.2)	55.4(2.9)	53.9 (3.3)
Above-ground biomass	27.8(2.1)	23.7(2.0)	45.2 (2.1)	37.8(2.2)	28.8(2.0)	27.6(2.0)	45.7(2.0)	38.2(2.0)	27.6(2.0)	25.9(2.1)	42.3(2.4)	36.3(1.8)	33.2(2.0)	25.6(2.1)	44.3(1.9)	32.2(2.2)
Lignotuber:stem	0.9(0.1)	0.7(0.1)	1.1 (0.1)	0.8(0.1)	0.8(0.1)	0.8(0.1)	0.8(0.1)	0.9(0.1)	0.8(0.1)	0.8(0.1)	1.1(0.2)	0.8(0.1)	1.2(0.1)	1.3(0.1)	1.5(0.1)	1.4(0.1)
Leaf water content	29.0 (0.8)	32.9(0.7)	29.2(0.7)	27.9(0.8)	29.4(0.7)	34.8(0.7)	29.9(0.7)	28.3(0.7)	27.9(0.7)	33.4(0.8)	28.7(0.9)	27.4(0.7)	28.2(0.7)	34.0(0.8)	29.6(0.7)	28.2 (0.8)
Nitrogen	1.8(0.1)	2.0(0.1)	1.9(0.1)	2.3(0.1)	1.7(0.1)	1.8(0.1)	1.7(0.1)	2.2(0.1)	2.0(0.1)	1.9(0.1)	2.0(0.1)	2.3(0.1)	1.9(0.1)	2.0(0.1)	1.9(0.1)	2.4(0.1)
Carbon	44.8(0.2)	44.7(0.2)	45.3(0.2)	45.3(0.2)	45.1(0.2)	45.3(0.2)	45.6(0.2)	45.5(0.2)	44.5(0.2)	44.9(0.2)	45.1(0.2)	45.4(0.2)	43.8(0.2)	44.4(0.2)	44.4(0.2)	44.7 (0.2)
1,8-Cineole	24.9(1.0)	24.6(1.0)	22.8(1.0)	21.6 (1.1)	26.4(1.0)	25.4(1.0)	23.7(1.0)	23.0(1.0)	23.8(1.0)	25.9(1.0)	22.8(1.2)	22.2(0.9)	22.5(1.0)	23.3(1.0)	21.5(0.9)	20.4 (1.1)
α-Pinene	6.9(0.5)	6.5(0.5)	7.2(0.5)	7.5(0.5)	7.0(0.5)	6.6(0.5)	7.4(0.5)	7.3(0.5)	7.1(0.5)	6.4(0.5)	7.4(0.6)	7.4(0.4)	6.5(0.5)	6.0(0.5)	6.8(0.5)	6.4(0.5)
Aromadendrene	2.9(0.2)	2.8(0.2)	2.8(0.2)	2.7 (0.2)	3.0(0.2)	3.3(0.2)	2.9(0.2)	2.8(0.2)	2.9(0.2)	2.9(0.2)	2.7(0.2)	2.7(0.2)	2.7(0.2)	2.8(0.2)	2.6(0.2)	2.5(0.2)
Globulol	1.7(0.13)	1.6(0.1)	1.6(0.1)	1.6(0.1)	1.8(0.1)	1. 9(0.1)	1.7(0.1)	1.7(0.1)	1.7(0.1)	1.7(0.1)	1.6(0.2)	1.5(0.1)	1.5(0.1)	1.6(0.1)	1.5(0.1)	1.4(0.1)
Limonene	2.0(0.2)	2.0(0.2)	1.8(0.2)	1.7(0.2)	2.3(0.2)	2.3(0.2)	1.8 (0.2)	2.1(0.2)	1.7(0.2)	2.2(0.2)	1.6(0.2)	1.6(0.2)	1.5(0.2)	1.8(0.2)	1.4(0.2)	1.3(0.2)
Total oil yield	47.7(2.3)	47.3(2.2)	44.8 (2.3)	45.2(2.4)	50.3 (2.2)	50.4(2.2)	46.2(2.3)	48.0(2.3)	46.1(2.2)	48.9(2.3)	44.9(2.5)	45.5(2.1)	40.9(2.2)	42.5(2.3)	40.3(2.1)	40.3(2.4)
Chlorogenic acid	2.3(0.4)	3.2(0.4)	2.9(0.4)	2.6(0.4)	3.5(0.4)	3.9(0.4)	3.6(0.4)	3.2(0.4)	3.6(0.4)	4.6(0.4)	3.8(0.4)	3.2(0.3)	3.7(0.4)	4.3(0.4)	4.5(0.3)	3.4(0.4)
Condensed tannins	1.1(0.9)	2.2(0.9)	0.6(0.9)	0.3(0.9)	3.9(0.9)	4.6(0.9)	1.9(0.9)	2.4(0.9)	2.7(0.9)	6.0(0.9)	1.7(1.0)	1.0(0.8)	1.2(0.9)	3.8 (0.9)	1.5(0.8)	2.2(0.9)
Total phenolics	228(9.8)	228(9.5)	228(9.4)	205(10.1)	253(9.4)	243(9.4)	228(9.8)	212(9.4)	214(9.4)	253(9.7)	224(11.1)	205(8.4)	217(9.4)	239(9.7)	218(8.6)	189(10.1)
Late-eluting FPCs	30.8(2.4)	25.6(2.5)	31.7(2.4)	30.8(2.5)	29.8(2.4)	27.1(2.3)	34.2(2.4)	29.5(2.4)	24.8(2.3)	23.8(2.4)	26.7(2.6)	26.0(2.3)	23.3(2.3)	19.5(2.5)	23.1(2.2)	23.9(2.5)
Macrocarpal A	2.5 (0.2)	2.0(0.3)	2.3(0.2)	2.0(0.2)	2.7(0.2)	2.4(0.2)	2.7(0.2)	2.3(0.2)	2.0(0.2)	2.0(0.2)	1.7(0.2)	1.8(0.2)	2.0(0.2)	1.7(0.2)	1.7(0.2)	1.8(0.2)
Macrocarpal G	8.4(0.7)	6.8(0.8)	7.5(0.7)	6.8(0.7)	8.5(0.7)	7.9 (0.7)	8.7(0.7)	7.6(0.7)	6.8(0.7)	6.3(0.7)	5.9(0.8)	5.9(0.7)	6.8(0.7)	5.6(0.7)	5.7(0.7)	6.2(0.8)

[3]*LMA* Leaf mass per area expressed in $g.m^{-2}$. Above-ground biomass expressed in g^{-1} DW. Lignotuber:stem is a diameter ratio. Leaf water content, nitrogen and carbon expressed as % DW. Concentrations of 1,8-cineole, chlorogenic acid and macrocarpal A expressed as mg g^{-1} DW. Concentrations of α-pinene, aromadendrene, globulol, total oil and limonene expressed as mg g^{-1} DW cineole equivalents. Concentrations of macrocarpal G and the late-eluting FPCs expressed as mg g^{-1} DW macrocarpal A equivalents. Concentrations of condensed tannins expressed as mg g^{-1} DW sorghum tannin equivalents, and total phenolics as mg g^{-1} DW gallic acid equivalents. Grey shading used to differentiate localities. LSmean (±SE) data in supplemental tables S1 and S2 taken from a mixed model using the full interaction of main effects (species×locality×treatment×harvest), refer to section '*statistical analysis*' for details.

Supplemental Table S4. Least squares means (±SE) of traits from juvenile ***Eucalyptus viminalis*** grouped localities (King island, Southern Tasmania, Queens Domain, St Helens), sampling period (Harvest1 or two weeks after at Harvest 2) and treatments (control, water limited/recovery)[4]

	King island				Southern Tasmania				Queens Domain				St Helens			
	Harvest 1		Harvest 2		Harvest 1		Harvest 2		Harvest 1		Harvest 2		Harvest 1		Harvest 2	
	Control	*Water limited*	*Control*	*Recovery*	*Control*	*Water limited*	*Control*	*Recovery*	*Control*	*Water limited*	*Control*	*Recovery*	*Control*	*Water limited*	*Control*	*Recovery*
LMA	65.1(3.1)	72.5(3.1)	58.4(3.1)	58.9(3.1)	74.1(3.2)	73.2(3.3)	58.8(3.2)	54.8(3.1)	69.1(3.1)	76.8(3.2)	57.7(3.0)	53.4(3.3)	70.7(3.3)	72.5(3.3)	58.9(3.4)	57.0(3.1)
Above-ground biomass	22.1(2.0)	21.2(2.0)	28.8(2.0)	29.9(2.0)	21.2(2.0)	17.7(2.1)	29.1(2.1)	23.7(2.0)	21.2(2.0)	19.0(2.1)	27.9(1.9)	23.6(2.2)	23.9(2.1)	21.5(2.2)	34.0(2.2)	27.8(2.0)
Lignotuber:stem	0.7(0.1)	0.8(0.1)	0.9(0.1)	0.9(0.1)	1.0(0.1)	1.0(0.1)	1.1(0.1)	1.1(0.1)	1.2(0.1)	1.3(0.1)	1.4(0.1)	1.5(0.1)	1.4(0.1)	1.3(0.1)	1.5(0.1)	1.5(0.1)
Leaf water content	32.1(0.7)	35.7(0.7)	31.1(0.7)	30.9(0.7)	32.8(0.7)	37.3(0.8)	31.9(0.8)	31.3(0.7)	33.5(0.7)	37.6(0.8)	31.4(0.7)	30.0(0.8)	33.9(0.8)	37.0(0.8)	31.3(0.8)	30.6(0.7)
Nitrogen	2.3(0.1)	2.4(0.1)	2.4(0.1)	2.5(0.1)	2.3(0.1)	2.4(0.1)	2.5(0.1)	2.9(0.1)	2.4(0.1)	2.3(0.1)	2.6(0.1)	3.1(0.1)	1.9(0.1)	2.2(0.1)	2.3(0.1)	2.6(0.1)
Carbon	48.2(0.2)	48.2(0.2)	48.9(0.2)	48.7(0.2)	48.0(0.2)	47.7(0.2)	48.8(0.2)	49.1(0.2)	47.7(0.2)	47.7(0.2)	48.8(0.2)	48.7(0.2)	47.7(0.2)	48.0(0.2)	48.8(0.2)	48.8(0.2)
1,8-Cineole	24.9(1.0)	23.9(1.0)	21.9(1.0)	20.9(1.0)	24.7(1.0)	24.3(1.1)	22.1(1.1)	21.2(1.0)	23.1(1.0)	23.0(1.0)	20.2(1.0)	18.6(1.1)	23.6(1.0)	21.9(1.1)	20.8(1.1)	20.5(1.0)
α-Pinene	7.7(0.5)	7.4(0.5)	8.1(0.5)	7.2(0.5)	6.7(0.5)	6.3(0.5)	7.3(0.5)	7.2(0.5)	5.7(0.5)	5.8(0.5)	6.4(0.5)	6.4(0.5)	5.6(0.5)	6.2(0.5)	6.3(0.5)	6.5(0.5)
Aromadendrene	3.6(0.2)	4.2(0.2)	3.5(0.2)	3. 9(0.2)	3.3 (0.2)	3.1(0.2)	3.5 (0.2)	3.6(0.2)	3.5(0.2)	3.5(0.2)	3.6(0.2)	4.1(0.2)	3.4(0.2)	3.6(0.2)	3.4(0.2)	3.6(0.2)
Globulol	2.0(0.1)	2.5(0.1)	2.1(0.1)	2.3(0.1)	1.9(0.1)	1.9(0.1)	2.1(0.1)	2.1(0.1)	2.3(0.1)	2.1(0.1)	2.1(0.1)	2.3(0.1)	2.0(0.1)	2.2(0.1)	2.0(0.1)	2.2(0.1)
Limonene	2.6(0.2)	2.8(0.2)	1.9(0.2)	2.0(0.2)	2.5(0.8)	2.4(0.2)	2.1(0.2)	2.0(0.2)	2.2(0.2)	2.4(0.2)	1.8(0.2)	1.5(0.2)	2.2(0.2)	2.2(0.2)	1.7(0.2)	1.6(0.2)
Total oil yield	62.5(2.2)	63.0(2.3)	56.2(2.2)	56.7(2.2)	57.5(2.2)	54.8(2.4)	58.9(2.3)	58.9(2.2)	57.5(2.2)	54.6(2.3)	54.4(2.1)	53.5(2.4)	51.0(2.3)	51.9(2.4)	52.0(2.4)	52.9(2.2)
Chlorogenic acid	1.5(0.4)	1.7(0.4)	1.5(0.4)	1.0(0.4)	3.0(0.4)	2.9(0.4)	2.3(0.4)	2.0(0.4)	3.2(0.4)	3.8(0.4)	3.1(0.3)	2.1(0.4)	2.7(0.4)	1.9(0.4)	2.4(0.4)	1.5(0.4)
Condensed tannins	10.3(0.9)	12.0(0.9)	6.7(0.9)	7.5(0.9)	6.9(0.9)	8.0(0.9)	5.5(0.9)	5.6(0.9)	8.4(0.9)	10.4(0.9)	7.0(0.9)	5.6(0.9)	6.0(0.9)	5.7(0.9)	5.2(1.0)	4.2(0.9)
Total phenolics	169(9.5)	181(9.5)	161(9.5)	161(9.5)	212(9.5)	212(10.2)	185(10.2)	159(9.2)	188(9.5)	210(9.8)	165(8.9)	146(10.2)	240(9.8)	225(10.2)	197(10.7)	180(9.8)
Late-eluting FPCs	34.4(2.3)	33.2(2.3)	31.2(2.3)	31.2(2.4)	24.2(2.3)	23.0(2.5)	25.3(2.4)	27.2(2.5)	26.8(2.3)	25.7(2.4)	28.1(2.3)	25.9(2.6)	30.1(2.5)	22.7(2.6)	27.9(2.5)	25.4(2.5)
Macrocarpal A	2.7(0.2)	2.3(0.2)	2.3(0.2)	2.3 (0.2)	1.7(0.2)	1.4(0.2)	1.7(0.2)	1.8(0.2)	1.9(0.2)	2.0(0.2)	2.1(0.2)	1.7(0.3)	2.5(0.2)	1.8(0.3)	2.1(0.2)	1.6(0.2)
Macrocarpal G	8.6(0.7)	7.2(0.7)	7.3(0.7)	7.7(0.7)	5.3(0.7)	4.2(0.8)	5.8(0.7)	6.1(0.7)	6.2(0.7)	6.1(0.7)	7.0(0.7)	6.2(0.8)	7.9(0.7)	5.3(0.8)	6.8(0.7)	5.6(0.8)

[4] *LMA* Leaf mass per area expressed in $g.m^{-2}$. Above-ground biomass expressed in g^{-1} DW. Lignotuber:stem is a diameter ratio. Leaf water content, nitrogen and carbon expressed as % DW. Concentrations of 1,8-cineole, chlorogenic acid and macrocarpal A expressed as mg g^{-1} DW. Concentrations of α-pinene, aromadendrene, globulol, total oil and limonene expressed as mg g^{-1} DW cineole equivalents. Concentrations of macrocarpal G and the late-eluting FPCs expressed as mg g^{-1} DW macrocarpal A equivalents. Concentrations of condensed tannins expressed as mg g^{-1} DW sorghum tannin equivalents, and total phenolics as mg g^{-1} DW gallic acid equivalents. Grey shading used to differentiate localities. LSmean (±SE) data in supplemental tables S1 and S2 taken from a mixed model using the full interaction of main effects (species×locality×treatment×harvest), refer to section '*statistical analysis*' for details.

Supplemental Table S5. Results of likelihood ratio test for variation of chemical and morphological traits in juvenile *Eucalyptus globulus* and *E. viminalis* leaves among mother trees (seed source) within localities (KI, ST, QD or SH), water treatments (control, limited water), sampling period (Harvest 1 and 2) and all interactions[5]

	Eucalyptus globulus								***Eucalyptus viminalis***							
	M (L)		H x M(L)		T x M(L)		H x T x M(L)		M (L)		H x M(L)		T x M(L)		H x T x M(L)	
	Chi^2	***P***	**Chi^2**	***P***	**Chi^2**	***P***	**Chi^2**	***P***	**Chi^2**	***P***	**Chi^2**	***P***	**Chi^2**	***P***	**Chi^2**	***P***
LMA	0.6		0.0		0.0		0.0		0.1		0.0		0.2		0.2	
Above-ground biomass	0.0		0.0		0.0		0.0		0.1		0.0		2.6		2.6	
Lignotuber/Stem ratio	1.6		1.8		0.0		0.0		0.8		1.9		0.0		0.0	
Leaf water content	0.0		0.0		0.0		0.0		0.0		0.0		0.6		0.6	
C:N	0.0		0.0		0.0		0.0		0.1		0.4		0.0		0.0	
1,8-cineole	0.3		1.6		0.0		0.0		0.0		0.4		0.0		0.0	
α-Pinene	0.3		0.5		0.0		0.0		0.3		1.0		0.0		0.0	
Aromadendrene	2.9		0.0		1.3		1.3		0.1		0.9		0.0		0.0	
Globulol	2.2		0.0		2.7		2.7		0.1		0.0		0.0		0.0	
Limonene	0.9		0.3		0.3		0.3		1.6		0.0		0.0		0.0	
Total oil	6.1	**#**	0.0		0.0		0.0		7.0	**#**	0.0		0.0		0.0	
Chlorogenic acid	0.0		0.1		0.0		0.0		2.0		0.0		0.0		0.0	
Condensed Tannins	0.1		0.0		0.0		0.0		2.1		1.0		0.0		0.0	
Total phenolics	0.0		0.0		0.0		0.0		0.1		0.0		0.0		0.0	
Other non-polar FPCs	12.5	*******	0.2		0.0		0.0		5.5	**#**	0.5		0.0		0.0	
Macrocarpal A	12.3	*******	0.0		0.0		0.0		9.1	******	0.0		0.0		0.0	
Macrocarpal G	10.5	******	0.5		0.0		0.0		10.8	*******	0.0		0.0		0.0	

[5] * indicates $P \leq 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$. *M* mother, *L* locality, *S* species, *H* harvest, *T* treatment. # indicates significant difference ($P \leq 0.05$) after *post hoc* tests (Tukey's) but no longer significant following control for false discovery rate (Benjamini & Hochberg 2000). Blank P-value indicates not significant ($P > 0.05$) *LMA* leaf mass per unit area, *KI* King Island, *ST* Southern Tasmania, *QD* Queens Domain, *SH* St Helens. *Lignotuber:stem* lignotuber diameter in relation to stem diameter expressed as a ratio. *C:N* the ratio of carbon to nitrogen. Species were analysed separately.

Supplemental Table S6. Least squares mean (LSmean) values (±SE) of morphological and chemical traits in juvenile *Eucalyptus globulus* from five localities[6]

Trait	**Locality**				
	JN	SH	QD	ST	KI
Above-ground biomass[1]	156.9 (11.9)^{a}	174.7 (14.3)^{ab}	242.6 (20.3)^{b}	229.2 (20.3)^{b}	180.9 (11.7)^{ab}
Leaf water content[2]	58.6 (0.5)^{ns}	60.6 (0.7)^{ns}	60.1 (0.9)^{ns}	60.0 (0.9)^{ns}	60.2 (0.5)^{ns}
Carbon[3]	45.3(0.1)^{a}	43.9(0.1)^{c}	44.4(0.2)^{bc}	44.7(0.2)^{b}	44.1(0.1)^{bc}
Nitrogen[3]	1.6(0.1)^{ns}	1.5(0.1)^{ns}	1.5(0.1)^{ns}	1.5(0.1)^{ns}	1.5(0.0)^{ns}
C:N	29.6(1.0)^{ns}	31.2(1.2)^{ns}	30.0(1.7)^{ns}	30.8(1.7)^{ns}	29.8(1.0)^{ns}
1,8-Cineole[5]	29.3 (1.3)^{a}	19.8 (1.5)^{b}	26.8 (2.1)^{a}	30.9 (2.1)^{a}	27.1 (1.2)^{a}
α-Pinene[6]	7.2 (0.4)^{a}	5.6 (0.5)^{b}	7.2 (0.6)^{ab}	8.3 (0.6)^{a}	7.5 (0.4)^{a}
Aromadendrene[6]	2.3 (0.1)^{a}	2.5 (0.2)^{a}	2.9 (0.2)^{a}	4.0 (0.2)^{b}	2.5 (0.2)^{a}
Globulol[6]	1.5 (0.1)^{a}	1.5 (0.1)^{a}	1.8 (0.1)^{a}	2.5 (0.1)^{b}	1.7 (0.1)^{a}
Limonene[6]	3.1 (0.1)^{a}	1.6 (0.2)^{b}	2.5 (0.2)^{a}	3.3 (0.2)^{a}	2.6 (0.1)^{a}
Total oil yield[6]	55.4 (2.2)^{a}	36.1 (2.7)^{b}	49.8 (3.7)^{a}	60.7 (3.7)^{a}	49.3 (2.1)^{a}
Chlorogenic acid[5]	4.8(0.2)^{ab}	5.7(0.2)^{a}	4.2(0.3)^{bc}	4.2(0.3)^{bc}	3.9(0.2)^{c}
Condensed tannins[7]	5.4 (0.6)^{a}	6.3 (0.7)^{a}	5.6 (0.9)^{a}	3.7 (0.9)^{ab}	1.9 (0.5)^{b}
Total Phenolics[8]	280.4 (5.9)^{ns}	274.8 (7.1)^{ns}	268.8 (10.0)^{ns}	265.6 (10.0)^{ns}	277.4 (5.8)^{ns}
Macrocarpal A[5]	0.6 (0.1)^{a}	1.3 (0.1)^{b}	2.1 (0.2)^{c}	2.6 (0.2)^{c}	1.9 (0.1)^{c}
Macrocarpal G[9]	1.8 (0.3)^{a}	4.0 (0.4)^{b}	6.0 (0.5)^{c}	8.2 (0.5)^{d}	6.0 (0.3)^{c}
Late-eluting FPCs[9]	17.3 (1.0)^{a}	17.5 (1.2)^{a}	21.8 (1.7)^{ab}	30.5 (1.7)^{c}	25.7 (1.0)^{bc}

[6] Letters indicate significant difference ($P \leq 0.05$) between localities for each trait after Tukeys *post hoc* tests.
ns non-significant. *KI* King Island, *ST* Southern Tasmania, *QD* Queens Domain, *SH* St Helens, *JN* Jeeralang North. *C:N* carbon to nitrogen ratio. For units of each trait; [1] indicates (g^{-1} DM), [2] indicates (% FW), [3] indicates (% DM), [5] indicates (mg g^{-1} DW), [6] indicates (mg g^{-1} DW cineole equivalents), [7] indicates (mg g^{-1} DW sorghum tannin equivalents), [8] indicates (mg g^{-1} DW gallic acid equivalents) and [9] indicates (mg g^{-1} DW macrocarpal A equivalents).

Supplemental Table S7. The degree of quantitative change (%) to traits of juvenile *Eucalyptus globulus* and *E. viminalis* provided 50% of the water evapotranspired by control plants[7]

	Eucalyptus globulus					***Eucalyptus viminalis***				
Trait	Chapter 2	Chapter 3	Chapter 4	Chapter 5	Mean	Chapter 2	Chapter 3	Chapter 4	Chapter 5	Mean
LMA	.	**12.1**	.	.	12.1	.	**5.7**	.	.	5.7
Growth	**-8.1**	**-12.5**	.	3.9	-5.6	**-13.5**	**-10.3**	-6.8	.	-10.2
Leaf water content	0.1	**-7.2**	.	**-7.5**	-4.9	-4.3	**-5.7**	**-2.6**	.	-4.2
C:N	**-20.2**	-3.9	.	-5.6	-9.9	**-11.2**	-3.1	**-11.5**	.	-8.6
1,8-Cineole	5.7	1.5	.	7.7	5.0	52.1	-3.2	**5.9**	.	18.3
α-Pinene	5.5	-7.2	.	6.0	1.4	246.1	0.1	**8.6**	.	84.9
Aromadendrene	-17.9	2.7	.	-6.8	-7.3	-17.3	4.3	-0.4	.	-4.5
Globulol	-19.4	3.0	.	-2.7	-6.4	-16.4	6.5	2.4	.	-2.5
Limonene	13.71	12.5	.	7.8	11.3	42.9	3.7	5.9	.	17.5
Total oil	-2.2	2.2	.	10.0	3.3	46.5	-1.9	**4.8**	.	16.5
Chlorogenic acid#	.	22.3	.	-11.2	5.6	.	-0.8	-6.6	.	-3.7
Condensed Tannins#	-36.4	**87.9**	.	37.5	29.7	-26.5	**14.3**	-2.6	.	-4.9
Total phenolics#	**-18.2**	5.7	.	2.2	-3.4	**-15.1**	2.3	-4.3	.	-5.7
Late-eluting FPCs^#	.	**-11.6**	.	2.7	-4.5	.	**-9.5**	0.4	.	-4.6
Macrocarpal A^#	.	**-12.8**	.	-17.3	-15.1	.	**-14.1**	-6.2	.	-10.2
Macrocarpal G^#	-18.5	**-12.5**	.	-19.9	-17.0	-19.4	**-18.5**	-9.8	.	-15.9

[7] Data taken from the four experiments described in Chapters 2-5. Bold and underlined values indicate results which were reported as significant in the respective chapter, after the statistical analysis and false discovery rate correction described therein. The means column for each species (grey) is the average percentage of quantitative change in each trait due to the treatment across the four experiments. Negative values indicate the percentage that concentrations decreased from control levels, positive values indicate quantitative increases compared the controls of each species in the respective experiment. *LMA* leaf mass per area, *growth* above-ground biomass, italics indicate terpenes, # indicates phenol, ^indicates formylated phloroglucinol compound (FPC).